REMARKS

This Reply is responsive to the Office Action dated December 22, 1999. Entry of the foregoing and favorable reconsideration of the subject application in light of the following remarks, pursuant to and consistent with 37 CFR §1.112, are respectfully requested.

At the outset, claims 1 and 14 have been amended above. The amended claims specify that the methods recited in claims 1 and 14 may be performed in the absence of a feeder layer, and find support on page 16, line 23.

Turning now to the Office Action, applicants acknowledge with appreciation the indication that claim 24 is allowable on page 8 of the Office Action. Applicants respectfully request confirmation of allowability, however, because page 1 of the Office Action indicates that claim 24 was rejected.

Claims 2-5, 12, 13, 15-19 and 23 were rejected under 35 U.S.C. §112, first paragraph as allegedly containing subject matter that is not enabled by the specification. The Examiner presents several bases for making the rejection, all of which are traversed. These bases will be addressed in the order presented in the Office Action.

First, it is the Examiner's opinion that it would require undue experimentation to practice the claimed culture method because the specification fails to disclose the species from which the growth factors were derived. The Examiner relies on Chang et al. (1995) -- which teaches that human SCF had no proliferative effect on chick PGCs and that the action of SCF might be species-specific -- as alleged evidence that the level of experimentation

required to practice the invention would be undue absent a teaching of a specific species from which the growth factors should be derived.

Applicants respectfully submit that they did not find it necessary to specify from what species the growth factors were derived, because in Applicants' experience, the relevant growth factors from different species have comparable activities at essentially the same dosage levels when used in the disclosed combination of factors. For instance, Applicants have found that both chicken and human SCF work in concert with the other listed growth factors at comparable concentrations. This suggests that growth factors such as these are highly conserved across species, and that the growth factors from one species will function to encourage the cells from another species to proliferate so long as the proper combination of factors is found. In fact, the growth factors currently being used by the inventors to maintain the cells were all derived from human, and are all commercially available.

It is pertinent to emphasize that the present inventors did not achieve the disclosed combination of growth factors merely by combining together factors which were known to affect avian cells. Rather, they started by taking factors that were known in the art to play a role in ES cell maintenance and were known at the time (each had been isolated from a species other than avian), and painstakingly tested combinations of these factors before they found a combination that was able to support the proliferation and maintenance of avian PGCs. In fact, none of these factors alone would work to support the proliferation of avian PGCs, so it is not surprising that Chang et al. reported no affect of human SCF on chicken

cells. Rather, it is the combination of growth factors that is important here, and Chang et al. did not utilize this combination.

It is also quite significant that the disclosed combination of growth factors facilitates proliferation and maintenance of PGCs without a feeder layer (see the specification, page 16, lines 21-25). Although the feeder layer of Chang et al. might have provided some of the other necessary factors for the chicken ES cells to proliferate, it is known in the art that feeder layers can also express inhibitory factors that deter proliferation. The culture system used by Chang et al. included a feeder layer, therefore the fact that they observed no affect of human SCF on the chicken cells does not necessarily mean that human SCF had no affect. It is a reasonable conclusion that Chang's feeder layer was inhibitory to the function of human SCF in that context, particularly given that Applicants found that human SCF works quite well in combination with the other disclosed factors to facilitate the proliferation of avian PGCs in the absence of a feeder layer. Reconsideration and withdrawal of this basis for the rejection is respectfully requested.

The claims were also rejected under the enablement prong of §112, first paragraph for failing to recite the "optimal" concentrations of growth factors as disclosed in the specification, because the Examiner believes that it would require undue experimentation to use concentrations above or below the most optimal. Applicants respectfully submit that the claimed ranges provide reasonable, not strict, boundaries for practicing the claimed invention, and the method need not be practiced at its optimum to be enabled. Furthermore, the quantity of growth factors required may vary depending on how many

cells per volume are cultured, and how fast they divide (for example, see page 19, lines 13-16 of the specification).

The Federal Circuit has explicitly stated that, "all experiments falling within the claim need not be optimal under all conditions for a valid patent." Atlas Power Co. v. E.I.DuPont de Nemours & Co., 224 USPQ 409, 414 (Fed. Cir. 1984). Therefore, it would be inappropriate to require optimal concentrations to be inserted into the claims when the claimed method need not be practiced at such concentrations, particularly when the claims are to be read in light of the specification, and the specification provides reasonable guidance by which the skilled artisan could practice the method without undue experimentation. "Where a specification does not require a limitation, that limitation should not be read from the specification into the claims." Specialty Composites v. Cabot Corporation, 6 USPQ 2d 1601, 1605 (Fed. Cir. 1988). Withdrawal of this basis for the rejection is respectfully requested.

The claims were also rejected for lack of enablement under §112, first paragraph, because the specification allegedly fails to enable an EG cell containing a transgene, as well as a method for producing chimeric avians wherein the method requires transfection of EG cells. The Office Action places particular emphasis on the statement from the specification that "to date, the growth of PGCs in culture for prolonged periods to facilitate selection of transfected PGCs has not been achieved" (Office Action, page 4, and specification, page 6, lines 6-9). Further, the Examiner apparently alleges that transfection of the EG cells would require undue experimentation by noting that the transfection described in the specification only achieved an average of 1 in 50 PGC transfectants, and no stable transfected cell line

was developed (although the Office Action does not state that it would require undue experimentation to transfect PGCs themselves, so it is not clear if the rejection extends to PGCs as well as EG cells). Applicants respectfully traverse this basis for the rejection.

Applicants respectfully note that the discussion on page 6 of the specification to which the Examiner refers concerns the deficiencies of the prior art methods, which, as Applicants specifically point out, suffered in particular from the lack of long term culture methods for chicken ES and PGC cells. The prior art had not failed in producing chimeric or transgenic avians as evidenced by the use of retroviral vectors to transfect cells (page 6, line 6); rather, such avians were previously *difficult* to produce due to the inability to culture PGCs for prolonged periods. Thus, the goal of the present invention was not to produce a transgenic or chimeric avian per se as a novel invention. Rather, Applicants' goal was to develop a long term culture system for avian PGCs that would *facilitate* the production of transgenic and chimeric avians.

The fact that it was known in the art that transgenic chimeric birds could be made using transformed PGCs is evidenced by the attached article by Vick and Simkiss. According to the abstract, these authors isolated PGCs from avian embryos and transfected the PGCs using defective retroviruses. These transfected PGCs were then injected into recipient embryos to form chimeras, some of which were able to pass the foreign DNA on to their offspring. What this group did not show is the ability to maintain the PGCs for long term in culture prior to or after transfection, a technique that would *facilitate* transfection with a wide variety of vectors other than retroviruses, that would have allowed for selection of cells receiving the transfected DNA prior to chimera formation, which in

turn would have increased the number and type of chimeric birds that could have been isolated using this technique.

Moreover, the ability to culture PGCs for prolonged periods not only facilitates the isolation of transfected PGCs, but also facilitates the isolation of transfected EG cells, because EG cell lines are produced as a result of long term culture of PGCs (see specification, page 6, lines 20-29). Facilitation of a transfected EG cell necessarily follows facilitation of transfected PGCs, because transfected PGCs become transfected EG cells by the culturing process for which the present invention provides.

Thus, the Examiner has rejected claims directed to methods of making transgenic chimeric avians because of deficiencies in the prior art which Applicants have in fact overcome. Moreover, the Examiner has rejected claims directed to such methods on the basis that no transgenic chimeric avians were demonstrated in a working example, when in fact such animals had been made before, just with much more difficulty in the absence of the present invention, which provides for long term culture of avian PGCs.

In this regard, the Federal Circuit has long held that 35 USC §112 does not require a specific teaching of that which is already known to one of ordinary skill in the art. Case v. CPC International, Inc., 221 USPQ 196, 201 (Fed. Cir. 1984). Moreover, it is well-established that the "absence of working examples in specification is without significance, since examples are not necessary and even though [they] may provide added useful information, the test is whether an individual possessed of knowledge of one skilled in the art could practice invention without exercise of undue amount of experimentation." Ex Parte Nardi & Simier, 229 USPQ 79, 80 (Bd. App. 1986).

That Applicants report that only 1 in 50 PGCs was transfected is not fatal, because the enablement test does not preclude all experimentation, just that which is undue. The fact that some experimentation is necessary to achieve transfection is true for any new cell line or a new vector construct, and in fact was true of the prior art techniques. As evidenced by Vick and Simkiss, persons of ordinary skill were not deterred from making transgenic chimeric avians even though they could not culture PGCs more than a few days, even though they could not confirm or select transfected PGCs before they proceeded with injection of the cells, and even though they had to go through the entire process of hatching the chimeric birds and mating them to see if the transfection enabled germ line transmission. The fact that persons using prior art techniques still sought to make transgenic chimeric birds despite the fact that they did not have the benefit of Applicants' culturing method suggests that, even in this uncertain environment, the level of experimentation was not undue. Applicants fail to comprehend, then, how the presently claimed methods of making transgenic chimeric avians would require "undue" experimentation when the claimed methods only make the prior art methods easier, and the level of experimentation was not so inconceivably large to deter prior researchers from making transgenic chimeric birds in the absence of the benefits to be gained by Applicants' invention.

Never-the-less, Applicants have attached hereto photographs of EG cells expressing a green fluorescence protein (GFP) transgene, thereby evidencing that the cells are capable of being transfected. Withdrawal of this basis for the §112, enablement rejection is respectfully requested.

The claims were also rejected for lack of enablement under §112, first paragraph, because the specification allegedly "fails to disclose specific promoters or regulatory sequences operable in avian cells or the identity of therapeutic polypeptides which would have a utility within avian animals." Accordingly, it is the Examiner's opinion that it would require undue experimentation for one of ordinary skill in the art to transfect EG cells such that a therapeutic effect could be achieved *in vivo* or *in vitro*.

Applicants first note that the Examiner has apparently misinterpreted the specification with regard to the therapeutic utility that must be enabled, in that the Examiner interprets the goal of the invention as pertaining to the therapeutic treatment of avians, not the use of avians as an expression system to express therapeutically useful polypeptides. The specification discusses the utility of the transgenic and chimeric eggs and chickens of the invention at page 10, lines 13-19, where it is stated: "It is expected that these transgenic chimeric avians will be useful for the recovery of heterologous proteins, which can be recovered directly from the eggs of such chimeric transgenic avians, or from tissues and other bodily fluids. For example, such avians can be used for the production and recovery of therapeutic proteins and other polypeptides" (with emphasis). Thus, when the claims are read in light of the disclosure (as they must be), it is clear that one use of the transgenic eggs and chickens of the present invention is to provide an expression system for useful proteins.

In this regard, it has long been understood by those having any skill in the art that the recombinant production of proteins enables an alternative environment from which to isolate a protein of interest, and often provides much more protein, or a more ready source

of protein, than if the protein were to be isolated from its natural environment. Given that the common definition of "therapeutic" is "having or exhibiting healing powers,"[1] it should be clear to one having ordinary skill in the art upon reading the claims in light of the disclosure that "therapeutic" proteins, or those having medicinal value, are only one type of useful protein that could be produced and recovered using the avian expression system described.

Never-the-less, it should be noted that the uses of the eggs and avians produced by the present invention are limited only by one's imagination. Indeed, the Examiner has noticed that the invention could also be employed to express proteins that are beneficial to the avian in which they are produced. The present methods, however, are not directed to methods of using the eggs and avians of the invention, but rather to making them. As such, a single disclosed use is sufficient (although many more are indeed possible).

The Examiner also alleges that it would require undue experimentation for one of ordinary skill to express a recombinant protein in the transfected avian PGCs or EG cells, because the specification allegedly fails to disclose specific promoters or regulatory sequences or vector construct design that would enable the recombinant expression of proteins. Applicants respectfully reiterate that the goal of the invention was not to enable transfection per se, because this had already been done. Nor was the goal to enable an avian expression vector, because these were available in the art, i.e. avian retroviral vectors, for instance. Nor was the goal to identify promoters and regulatory sequences that provide optimal expression of recombinant genes in avian cells, because those of skill in the

[1] See The American Heritage College Dictionary, 3rd ed. (1997), page 1406.

use of expression systems know fully well how to design their own promoter-driven gene constructs, having learned in a basic genetics course that the promoter used to express a recombinant protein in a heterologous cell must be capable of being recognized by the cellular machinery from the specific cell or animal to be used. Moreover, useful promoters for avians were known in the art as evidenced by Mahr and Ordahl (1988) (attached), which discloses an avian muscle promoter.

Rather, the goal of the present invention was to devise a culture medium for avian PGCs and EG cells to *facilitate* the transfection of cells, to *facilitate* the design of expression vectors, and to *facilitate* the design and use of promoters in such cells for the recombinant production of proteins, in that such culture medium facilitates the long term proliferation and maintenance of such cells without a feeder layer. As discussed beginning on page 16 of the specification, line 21, for instance, a particular advantage of the absence of a feeder layer is that it provides for purer colonies and a cleaner preparation when producing chimeric or cloned animals. It is the ability to culture PGCs long term without having to worry about adverse affects of the feeder layer on transgene expression, or adverse affects of the transgene on feeder layer function, which provides the point of novelty, not the ability to transfect the cells per se. In view of these comments, reconsideration and withdrawal of these bases for the rejection is respectfully requested.

The next basis for the §112, first paragraph enablement rejection is that the specification allegedly fails to enable a method of purifying a polypeptide from avian eggs, circulatory systems, bodily fluids or tissues. In particular, the Examiner points to a passage from a PTO hearing discussing how protein purification schemes vary widely from

protein to protein. Applicants respectfully submit that the method of the present invention is not directed to the purification of any specific protein, but rather to a culturing method for PGCs and EG cells in a precise combination of growth factors in the absence of a feeder layer as to facilitate transfection, chimera production and the recombinant production of proteins in transgenic animals. As such, the claimed method (particularly that covered by claim 19 which includes a protein purification step) would encompass *any* protein purification scheme *in general* whereby the person uses applicants' culturing technique.

This does not mean that a person who uses the instant culturing method would not be able to obtain a separate patent for a *specific* protein purification method if it was indeed novel. In that context, it is evident why such a person would need to enable the protein purification process, because that process would be the point of novelty. Indeed, such a process could likely be used to isolate the specific protein from any cell which expressed it, and would extend to the uses of cells and culturing methods that have nothing to do with the methods claimed here.

In contrast, Applicants seek to patent a culturing method. In that context, Applicants need not prove or show that any particular protein can be isolated from cells, blood or eggs, because it has been known for decades that proteins in general can be isolated from living cells. It follows then, that the cells obtained by Applicants' culturing method could be used to isolate proteins, and Applicants have every right to protect their specific culturing process as it is used within the general context of protein purification.

Applicants do not claim a process of purifying any particular protein, so the specification need not demonstrate the purification of particular proteins. Rather,

Applicants claim that the avian PGCs, eggs, and birds isolated by the culturing process may be used in general for the recombinant production of proteins. As such, it need only be clear to one of ordinary skill in the art that it is possible to isolate proteins from eggs and avians in general absent undue experimentation, and there were many references available in the art at the time of the invention which demonstrated that this is possible. Because the specification need not disclose that which is well known in the art in order to be enabling, it follows that the instant specification need not disclose the purification of proteins from eggs or avians.

For instance, Alexander and Collins reported in 1977 that infectious bronchitis virus could be propagated and isolated from eggs using sucrose density gradient centrifugation, and the viral polypeptides thereafter separated using SDS-PAGE. In 1993, Vieira and Schneider reported a one-step chromatographic method for the purification of serotransferrin from the serum of ovulating chickens. Both articles are attached to this Reply. Thus, it was known for at least two decades that proteins in general could be purified from eggs and from the bodily fluid of avians, and therefore Applicants need not enable general methods of protein purification because such methods were known in the art. Reconsideration and withdrawal for this basis for the rejection is respectfully requested.

The last basis for the lack of enablement rejection is that the specification allegedly fails to provide an enabling disclosure for the use of a Gallinacea or turkey PGC, because the specification only exemplifies the use of chicken PGCs , ES's and EG's. The Examiner further alleges that the results of one species cannot be applied directly to another species, and again cites Chang et al. as support.

Applicants again respectfully submit that Chang et al. is of no consequence here because Chang did not employ the disclosed combination of growth factors without a feeder layer. In fact, Applicants use human growth factors to propagate the chicken PGCs, evidencing that the requisite homology of these factors is sufficient for cross-species use. If human growth factors support the use of chicken PGCs it is reasonable to assume that they will also support the proliferation of turkey PGCs. Applicants have provided the requisite teaching that the disclosed combination of growth factors may be used to maintain PGCs without a feeder layer. Applicants have done the difficult work. One reading the patent could readily apply the combination to other species absent undue experimentation with the knowledge of Applicants' success in hand. Reconsideration and withdrawal of this basis for the rejection in view of these considerations is respectfully requested.

Claims 1-3, 13 and 14 were rejected under 35 U.S.C. §112, second paragraph for alleged indefiniteness. Specifically, claims 1-3 were rejected for the terms "sufficient," "minimal" and "maximal" concentrations of culture medium being used rather than "initial" and "optimal," and for failing to recite the species from which the growth factors were derived. Applicants respectfully submit that the term "sufficient" should not be read in a vacuum, and achieves clarity when it is read in context of the entire claim, i.e., "sufficient to produce a culture having a compact multilayer like appearance." Those of skill in the art of producing and maintaining EG cells know what culture appearance to strive for. "Minimal" and "maximal" were not used in the claims to denote a series of concentrations in a culturing experiment (for which the term "initial" might be more appropriate), but rather a range of concentrations that may be used for any particular cells. Applicants believe this is

clear when the claims are read in light of the specification, i.e., see page 19, lines 1-12. Moreover, claims 2 and 3 taken together do recite the optimal range (not strict limits), as Applicants have found that a lower concentration may also be used to support the proliferation and maintenance of cells. Withdrawal of the rejection as it applies to claims 1-3 is respectfully requested.

Claim 13 was rejected for the term "therapeutic" because it is allegedly not clear whether the term refers to a protein that treats or prevents an avian disorder, or a growth factor that facilitates cell growth and differentiation. As discussed above, the term "therapeutic" would be clear when read in light of the specification, and has a clear meaning in the art. Hence, it would be clear that "therapeutic" proteins are those which have or exhibit medicinal power, either for the avian in which they are produced or for a subject to which they are subsequently administered. "Therapeutic" would not be understood to encompass a growth factor that facilitates cell growth in culture, although such a growth factor might be considered "therapeutic" if used for the treatment of a subject in need thereof. Never-the-less, the instant culturing method encompasses the expression of any recombinant protein, and the cells may be engineered to express the recited growth factors for the purpose of facilitating cell growth in culture as an alternative to providing exogenous growth factor in the culture medium (see the specification, page 17, lines 3-9, for instance).

Finally, claim 14 was rejected because of the term "tissue" culture medium. "Tissue culture" has a standard and well-known definition in the art, and moreover, the claim makes it abundantly clear that the claimed medium is for culturing avian PGCs and EG cells.

Withdrawal of the rejection under 35 U.S.C. §112, second paragraph is respectfully requested.

Claims 1, 5-11, 14, 16 and 20-22 were rejected under 35 U.S.C. §103(a) as being unpatentable over Pain et al. in view of Labosky et al. Essentially, it is the Examiner's opinion that the claimed methods of generating EG cells and chimeric animals by culturing PGCs in the recited growth factors would be obvious in view of Pain et al., which allegedly teaches the long term maintenance of ES cells, but not PGCs, in the recited growth factors, and Labosky et al. which teaches that murine PGCs cultured in SCF, LIF and bFGF allows the undifferentiation of PGCs into EG cells. Applicants respectfully traverse the rejection as it applies to the original claims, and particularly as it fails to apply to the claims as amended above.

The Examiner is correct to note that Pain et al. fails to disclose the long term maintenance in culture of pure PGCs. However, as discussed in the Reply filed on April 26, 1999 in parent application 08/905,773, the cells propagated in Pain et al. were not pure PGCs or pure ES cells; they were actually an unpure mixture of blastoderm cells, which are, although they might contain a small percentage of PGCs, as a whole quite different from PGCs. The Examiner is also correct to note that Labosky et al. report that EG cells may be isolated by exposing PGCs to growth factors (even though Labosky et al. does not use all the PGCs recited in the instant claims, i.e., Labosky does not use IGF). However, Applicants fail to see how it would be obvious for the skilled artisan to combine the two disclosures to arrive at the claimed invention when the two cited references pertain to entirely different cell types.

Moreover, Pain et al. did not only use a mixture of the growth factors recited in the claims. Pain et al. also employed IL-11 and antiretinoic acid monoclonal antibody, which prevents differentiation of the cells. Thus, even if the skilled artisan could reasonably combine Pain et al. and Labosky et al. to arrive at a medium containing the four recited growth factors for isolating and cultivating EG cells, one would also believe it necessary based on Pain et al. to add IL-11 and antiretinoic acid monoclonal antibody in order to prevent cell differentiation. In contrast, Applicants have discovered that avian PGCs may be maintained in long term culture and used to produce EG cells with only the four recited growth factors being required (other than basic essentials).

Finally, it is pertinent to note that both Pain et al. and Labosky et al. require feeder cells (see Pain, p. 2345, col. 2 first full paragraph, and Labosky et al., page 3198, col. 1, line 7). Applicants' culturing medium is capable of maintaining avian PGCs and EG cells in prolonged culture without differentiation in the absence of a feeder layer. This would not have been obvious in view of Pain et al. and Labosky et al. taken apart or together, particularly in view of the statement in Labosky et al. that the EG cells described therein "require feeder cells and serum factors" (emphasis added) Id.

As discussed above, the ability to culture the cells in the absence of a feeder layer presents significant advantages for the long term culture of PGCs because often feeder cells express unknown inhibitory factors that affect the maintenance of the PGCs and transfection

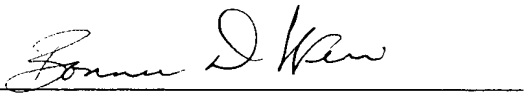
or expression of transgenes. Furthermore, feeder cells are an unwelcome source of contamination with respect to the generating of chimeric animals that can affect the efficiency of recovery of such animals. It would have been truly unexpected at the time the present invention was made, and even now, that PGCs could be made to proliferate for prolonged periods in culture in the absence of feeder cells.

[E]vidence of unobvious or unexpected advantageous properties may rebut a prima facie case of obviousness. In re Chupp, 2 USPQ2d 1437, 1439 (Fed. Cir. 1987). It would have been unobvious and unexpected that the recited growth factors could be used in combination to affect the long term growth of avian PGCs and EG cells, and particularly unexpected that such growth factors could be employed to maintain such cells in the absence of a feeder layer. Reconsideration and withdrawal of the §103(a) rejection based on Pain et al. and Labosky et al. is respectfully requested.

The above remarks appear to be fully responsive to the Office Action dated December 22, 1999. Therefore, a Notice of Allowance is next in order. If there are any questions regarding this amendment and response, or with the application in general, the Examiner is respectfully requested to telephone the undersigned so that prosecution of the present application may be expedited.

Respectfully submitted,

BURNS, DOANE, SWECKER & MATHIS, L.L.P.

By: 

Bonnie D. Weiss
Registration No. 43,255

P.O. Box 1404
Alexandria, Virginia 22313-1404
(703) 836-6620

Date: May 22, 2000

Exhibit 8 attachment
09/127,738

The purification and polypeptide composition of avian infectious bronchitis virus

D. J. Alexander and M. S. Collins

Poultry Department, Central Veterinary Laboratory, New Haw, Weybridge, Surrey KT15 3NB, Great Britain

Abstract

Purification of egg-grown infectious bronchitis virus (IBV) by sucrose density gradient centrifugation alone, or sucrose density gradient centrifugation plus pH 8.0 treatment, concanavalin A precipitation or metrizamide density gradient centrifugation, failed to produce any differences in the virus polypeptide pattern following polyacrylamide gel electrophoresis in the presence of SDS(SDS-PAGE). SDS-PAGE of purified IBV on 7.5% acrylamide gels separated 16 polypeptides which were detectable by staining with Coomassie blue or measurement of radioactivity following electrophoresis of (^{3}H)-leucine labelled IBV. The molecular weights of the polypeptides were within the range 15,000-135,000.

The polypeptides of egg and chick kidney (CK) cell-grown IBV were identical in both size and number but quantitative differences were detected. In particular the relative proportion of the major 52,000 molecular weight polypeptide was greatly reduced in IBV grown in CK cells. SDS-PAGE of purified IBV and staining with Schiff's reagent to detect carbohydrate revealed four bands with molecular weights of 128,000, 86,000, 67,500 and 37,000. The 128,000 band did not correspond to any of the detected polypeptides. Use of 5% acrylamide gels for SDS-PAGE of IBV failed to resolve all the minor polypeptides and only seven bands were detected.

Introduction

Studies on the polypeptide composition of different coronaviruses have produced conflicting results in respect to both the number and size of the polypeptides detected. Hierholzer *et al.* (1972) examined the protein composition of the human coronavirus OC 43 and detected 6–7 polypeptides with a molecular weight range of 15,000–191,000, four of which were glycopolypeptides. Garwes and Pocock (1975), working with transmissable gasteroenteritis virus (TGEV) of pigs, detected six polypeptides with a molecular weight range of 28,500–200,000 of which three were glycopolypeptides. These authors also report differences in the effect of bromelain on the composition of their respective viruses. However, the differences reported between OC43 and TGEV are relatively minor compared to differences in the number of polypeptides reported for avian infectious bronchitis virus (IBV). Bingham (1975) detected at least 16 polypeptides of which four were glycosylated, and Collins, Alexander and Harkness (1976) described at least 14 polypeptides of which four were glycopolypeptides. Although the two IBV reports show comparable polypeptide patterns, such large discrepancies with other coronaviruses must cast some doubt on the purity of the virus preparations used in these studies.

Possible sources of contamination of virus preparations include: soluble proteins in growth media, bacteria, mycoplasma and endogenous viruses, but most consideration must be given to the subcellular particles and cell debris produced during the virus growth cycle, particularly if the virus under study is cytopathic. Maximum purification of viruses by density gradient centrifugation is best obtained by the combination of rate zonal and equilibrium density gradient centrifugation since the probability that

extraneous cellular material is present, which has the same sedimentation co-efficient and buoyant density as the virus, is very small. Anderson *et al.* (1966) discuss such two dimensional separation of viruses in detail and have collated data that suggest that most virus particles fall into a 'virus window' when separated by both sedimentation co-efficient and buoyant density properties.

Other methods of virus purification have also involved selective sedimentation or precipitation. Metrizamide density gradient centrifugation may be used in conjunction with sucrose density gradients, since metrizamide has been shown to increase the hydration levels of nucleic acid and thus affect the density (Birnie, Rickwood and Hell, 1973). Viruses could therefore be separated from cellular material which does not contain nucleic acid but has the same density on sucrose gradients. Concanavalin A (Con A) precipitation has been used as a stage in the purification of oncornaviruses (Stewart *et al.*, 1973) and may serve as a method of separating viruses from some contaminating proteins.

Other considerations may also be important on the purification of coronaviruses. For example Pocock and Garwes (1975) have shown that at pH 7.2 or lower TGEV is associated with cell membrane material and have used pH 8.0 treatment as part of their purification procedure for this virus (Garwes and Pocock, 1975).

The present study was initiated to examine the purification of IBV by several procedures incorporating the different methods outlined above and to analyse the polypeptide content of the resulting purified virus.

Materials and methods

Radioisotope

L-(4,5-^{3}H)-leucine (sp.act.56 Ci/mmol) was obtained from The Radiochemical Centre, Amersham, Buckinghamshire, England.

Cell cultures

Monolayers of primary chicken kidney (CK) cells from 4-week-old specific pathogen free white leghorn chickens were prepared as described (Bracewell, 1975), and grown in 750 cm^2 roller culture bottles rotating at ten revolutions per hour.

Virus growth

The Beaudette strain of IBV was used throughout this study. Growth in eggs was as described (Collins *et al.*, 1976). Radioisotope-labelled virus was obtained by infecting confluent roller culture monolayers of CK cells with 5 Egg LD_{50}/cell, and 1 h later adding 20 ml of Hank's balanced salt solution containing amino acids and buffered to pH 6.5 with 15 mM N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES) to each 750 cm^2 bottle. The amino acids present and their concentrations were as listed by Garwes and Pocock (1975) except that leucine was omitted and replaced with (^{3}H)-leucine to give 10 μCi/ml.

Virus purification

Unless stated otherwise, all dilutions and solutions were made with 0.01 M Tris/HCl buffer at pH 6.5.

Virus was purified by one of four procedures: (1) sucrose density gradient equilibrium centrifugation. Infected allantoic or tissue culture fluids were clarified by low speed centrifugation and the virus pelleted at 20,000 *g* for 40 minutes. The virus pellet was resuspended at an approximately 80-fold concentration. The concentrated virus was then layered onto a 20 ml linear 20–50% w/w sucrose gradient and centrifuged for 20 min at 44,000 x *g*. The virus band from the gradient was then diluted and applied to a 20–55% w/w linear sucrose gradient and centrifuged for 18 h at 70,000 x *g*.

(2) Sucrose and metrizamide equilibrium density gradient centrifugation. The virus was treated as described for method (1), dialysed for 5 h to remove sucrose, layered on an 18 ml 0–50% w/v linear metrizamide (Nyegaard and Co. A/S, Oslo, Norway) gradient and centrifuged at 70,000 x *g* for 18 h. (3) Sucrose equilibrium centrifugation and concanavalin A (Con A) precipitation. The virus was treated as described for method (1) and further purified by precipitation and dissociation with Con A as described (Stewart *et al.*, 1973) followed by further sucrose equilibrium density gradient centrifugation. The virus suspension was made 1 mM with respect to manganese chloride and calcium chloride before Con A precipitation. (4) pH 8.0 treatment. Concentrated virus was resuspended in 0.01 M Tris/HCl pH 8.0 buffer, left for 2 h at 4°C, applied to a 20–50% w/w sucrose gradient in 0.01 M Tris/HCl pH 8.0 and centrifuged for 20 min at 44,000 x *g*. Virus from this gradient was then applied to a sucrose gradient at pH 6.5 for equilibrium centrifugation.

Polypeptide analysis

Polypeptides were analysed by PAGE as described (Alexander, 1974). For the detection of polypeptides, gels were stained overnight with 0.1% Coomassie brilliant blue in 50% v/v methanol 7.5% v/v acetic acid, and destained in a diffusion destainer (Bio Rad Laboratories, U.S.A.) containing 50% v/v methanol 7.5% v/v acetic acid. For the detection of carbohydrates, gels were fixed for 24 h with repeated changes of 15% v/v acetic acid, and then placed in a solution containing 1% w/v periodic acid and 3% v/v acetic acid for 3 h followed by repeated washing in distilled water for 5 h. Gels were then immersed in Schiff's reagent, and left in the dark overnight before destaining in 50% v/v methanol and 0.1% w/v sodium metabisulphite in 0.01 N HCl. To prepare Schiff's reagent 1.0 g basic fuchsin (Gurr Ltd) was dissolved in 200 ml of boiling distilled water. This was then cooled, filtered and 30 ml of 1 N HCl added, followed by 3.0 g potassium metabisulphite. The solution was allowed to bleach for 48 h in the dark at room temperature; it was then shaken with 2.0 g animal charcoal and used after refiltering.

Gels which received radioisotope-labelled virus were frozen, cut into 1 mm slices with a Mickle gel slicer (Mickle Laboratory Engineering Co., Gomshall, Surrey, England) and radioactivity estimated as described (Alexander and Reeve, 1972).

The molecular weights of the polypeptides were estimated by comparison of the migration following PAGE with standards of known molecular weight. The standards used were: bovine serum albumin, human gammaglobulin and the polypeptides of the Herts or Ulster strains of NDV using the polypeptide molecular weights reported for this virus by Moore and Burke (1975).

Infectivity titrations

Infectivity was estimated in eggs as described (Collins *et al.*, 1976).

Results

Virus purification

Rate zonal centrifugation of concentrated IBV on sucrose density gradients, whether at pH 6.5 or pH 8.0, produced a broad spread of virus with a peak at 1.150–1.155 g/cm^3 (Figures 1a and 1b).

Equilibrium centrifugation on sucrose gradients produced much sharper bands with most of the virus between 1.165 and 1.200 g/cm^3 with peaks at 1.175–1.180 g/cm^3 for virus from rate zonal gradients (Figures 1c and 1d).

Virus was detected at much lower densities on metrizamide gradients and produced a peak at 1.14 g/cm^3 (Figure 1e).

Con A treatment tended to increase the density of IBV on sucrose gradients so that a peak was detected at 1.20 g/cm^3. The infectivity of the virus was destroyed by Con A treatment.

Polypeptide analysis

SDS-PAGE under reduced conditions produced an identical polypeptide pattern for IBV regardless of the method of purification (Figure 2). The extra polypeptide (mw 31,000) seen in gels of Con A-treated virus probably relates to part of the Con A which remains bound after dissociation (Stewart *et al.*, 1973), and may be responsible for the loss in infectivity of treated virus.

Since no difference in polypeptide pattern was obtained by any of the purification procedures, virus for all further studies was purified by rate zonal and equilibrium centrifugation on sucrose gradients at pH 6.5.

More detailed examination of IBV polypeptides by SDS-PAGE and staining for polypeptides and carbohydrates revealed a total of 16 polypeptides (Figure 3). Four major polypeptides were present with molecular weights of 52,000, 47,000, 39,000 and 36,000 (Table 1). The 39,000 and 36,000 molecular weight polypeptides generally overlapped to some extent. Eight of the other polypeptides were consistently detected but the polypeptides of 135,000, 63,500, 32,000 and 15,000 molecular weights were not always apparent.

Staining for carbohydrate revealed four bands with molecular weights 128,000, 86,000 , 67,500 and 36,000 (Figure 3). All but the 128,000 band corresponded to Coomassie blue-stained polypeptides. Occasionally an area of slight staining was seen which corresponded to the 52,000 major polypeptide.

In an attempt to detect any polypeptides of sufficiently high molecular weight to be excluded from 7.5% acrylamide gels, polypeptides were analysed on 5% acrylamide gels. No polypeptides of higher molecular weight than those seen on 7.5% acrylamide gels were detected. Electrophoresis on 5% acrylamide gels also failed to separate the majority of the minor bands, so that only seven polypeptides were apparent with molecular weights 135,000, 102,000, 92,000, 74,000, 53,000, 47,000 and 40,000 (Figure 4).

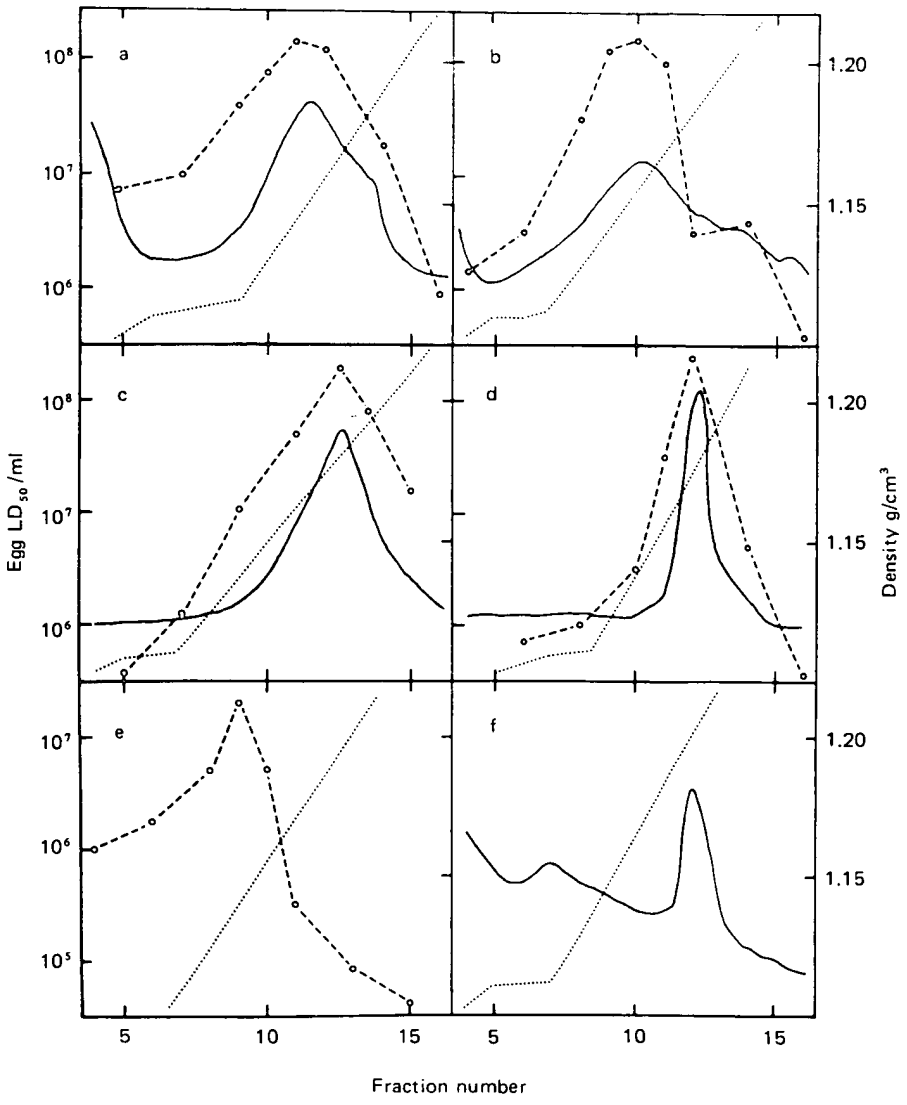


Figure 1 Density gradient centrifugation of IBV (a) and (b): rate zonal centrifugation on a linear 20–50% w/w sucrose gradient at 44,000 x *g* for 20 min. Sucrose and sample made up in 0.01 M Tris/HCl buffer at (a) pH 6.5 (b) pH 8.0.(c) Equilibrium centrifugation of virus from (a) on a linear 20–50% w/w sucrose gradient in 0.01 M Tris/HCl pH 6.5 at 70,000 x *g* for 18 h. (d) Equilibrium centrifugation of virus from (b). (e) Equilibrium centrifugation of virus from (c) on a linear 0–50% w/v metrizamide gradient at 70,000 x *g* for 18 h. (f) Equilibrium centrifugation of virus from (c) on a sucrose gradient after Con A treatment. O- - - O, Infectivity. E_{260}, unbroken line, and density, dotted line, graphs.

Table 1 Polypeptides of IBV compared with those of TGEV and mouse mammary tumour (MMTV)

Molecular weight $\times 10^{-3}$	Gel strength % acrylamide: 7.5 IBV	5.0 IBV	5.0 TGEV**	7.5 MMTV**
			200 G	
	135*	135*		
	102 G	102 G	105	
	92	92		90 G
	86 G	G		
	78		80.5	78
	74	74		
	68 G	G		68 G
	63.5* G			
	52	53	50	55 G
	47	47		42
	39	40		
	36 G	G		34 G
	32*		30 G	
	27		28 G	28
				23
	18			18
	15*			14
				12

G = Glycosylated. Bands on 5% acrylamide gels of IBV stained with Schiff's reagent did not necessarily correspond to bands on 5% gels stained with Coomassie blue. A carbohydrate-containing band of 128,000 molecular weight seen on gels of IBV stained with Schiff's reagent did not correspond to any band on gels stained with Coomassie blue.

* These bands were indistinct or not always present.

** Source — Garwes and Pocock (1975).

*** Source — Sarkar and Dion (1975).

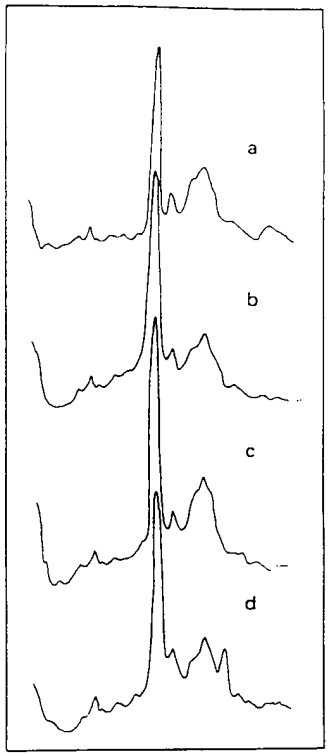


Figure 2 SDS-PAGE analysis of the polypeptides of IBV purified by different methods. (a) Sucrose density gradient equilibrium centrifugation. (b) pH 8.0 treatment. (c) Metrizamide density gradient equilibrium centrifugation. (d) Con A treatment. Gels were stained with Coomassie blue.

Comparison of egg and tissue culture grown virus

Comparison of egg and CK cell grown virus revealed no difference in the number or the molecular weights of polypeptides detected (Figure 5). However, virus grown in CK cells showed a substantial reduction in the concentration of the major 52,000 molecular weight polypeptide which represented 40% of the total protein in egg grown virus but only 20% in virus grown in CK cells.

Tissue culture-grown virus was also labelled with (^{3}H)-leucine and analysed by SDS-PAGE (Figure 5c). Although a similar number of polypeptides to those seen in stained gels were present, several quantitative differences were apparent, these may relate to the leucine content of the polypeptides. No peak of radioactivity corresponding to a polypeptide with molecular weight 128,000 was seen on either 7.5% or 5% acrylamide gels of (^{3}H)-leucine labelled virus.

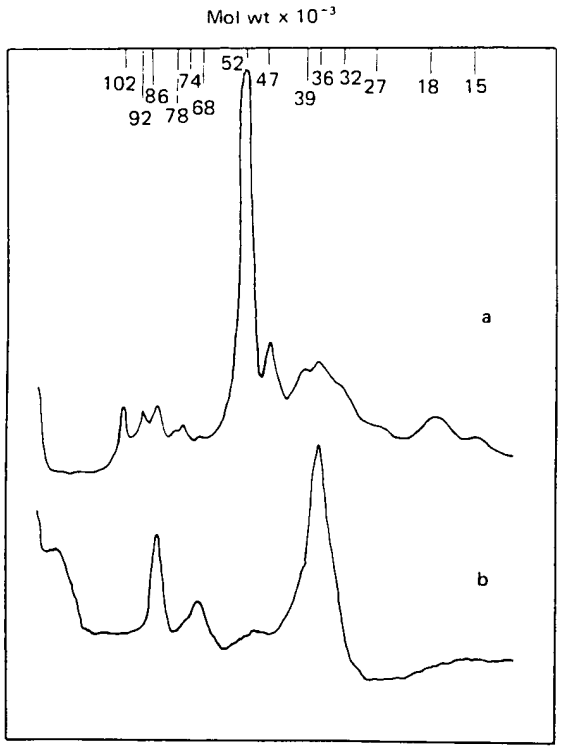


Figure 3 SDS-PAGE analysis of purified egg-grown IBV on 7.5% acrylamide gels. (a) Gels stained for polypeptides with Coomassie blue and scanned at 620 nm. (b) Gels stained for carbohydrate with Schiff's reagent and scanned at 520 nm.

Discussion

Our results show that the polypeptide composition of IBV purified by rate zonal and equilibrium density gradient centrifugation is identical to that of virus given additional treatment involving metrizamide density gradient centrifugation, Con A precipitation or pH 8.0 treatment. These findings suggest that the initial treatment is sufficient to produce highly purified virus and that IBV falls within the 'virus window' conceived by Anderson *et al.* (1966).

Centrifugation of IBV on metrizamide gradients showed that the virus has a lower density in the presence of metrizamide than in the presence of sucrose. This indicates the usefulness of metrizamide in virus purification since cell material, which has the same density as the virus on sucrose but does not contain nucleic acid, is unlikely to show a similar change in density on metrizamide.

Our results show that IBV has 16 apparent structural polypeptides ranging in molecular weight from 135,000 to 15,000 of which three with molecular weights 86,000, 67,500 and 36,000 contain carbohydrate. The additional band on gels stained with

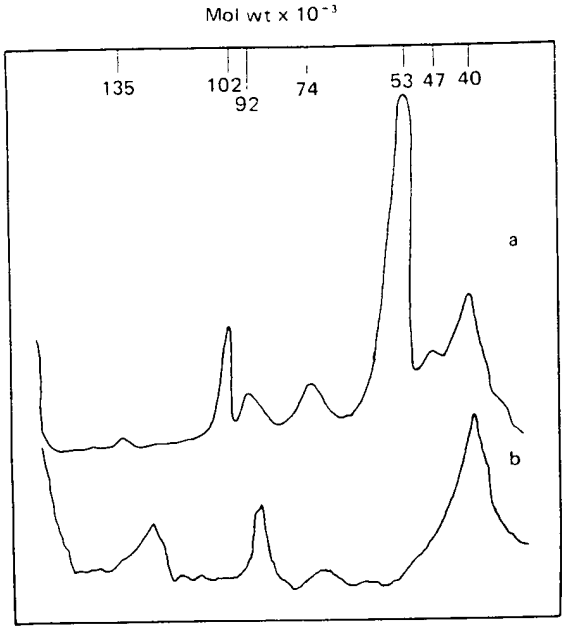
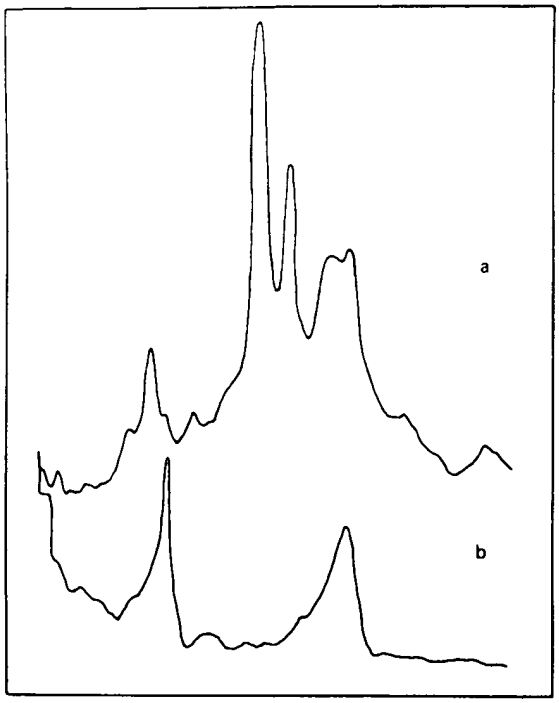


Figure 4 SDS-PAGE analysis of purified egg-grown IBV on 5% acrylamide gels. (a) Gels stained for polypeptides with Coomassie blue. (b) Gels stained for carbohydrate with Schiff's reagent.

Schiff's reagent did not correspond to any band stained with Coomassie blue, and may represent either a non-protein glycosylated macromolecule or alternatively contain a polypeptide not stained by Coomassie blue (Fairbanks, Steck and Wallach, 1971). However, the absence of a 128,000 mw peak in gels of (^{3}H)-leucine labelled IBV suggests that the former explanation is correct. The number of polypeptides detected and their glycosylation compare favourably with previous reports (Bingham, 1975; Collins *et al.*, 1976) and are well within the coding capacity of the IBV genome size reported by Watkins, Reeve and Alexander (1975). However, the polypeptides of IBV appear to be quite different, especially in the number present, to those of TGEV (Garwes and Pocock, 1975) or OC43 (Heirholzer *et al.*, 1972). One possibility is that host material is incorporated into IBV particles, as suggested by Berry and Almeida (1968), but not into other coronaviruses. Alternatively the similarity in number and some molecular weights of the major polypeptides of virus grown in CK cells and the polypeptides of TGEV grown in primary pig kidney cells may indicate a failure to detect the minor bands in studies with other coronaviruses. In this respect it may be significant that the minor polypeptides of IBV were not resolved on 5% acrylamide gels and only seven polypeptide bands were detected under these conditions (Figure 4). Garwes and Pocock (1975) used 5% acrylamide gels for TGEV polypeptide analysis.



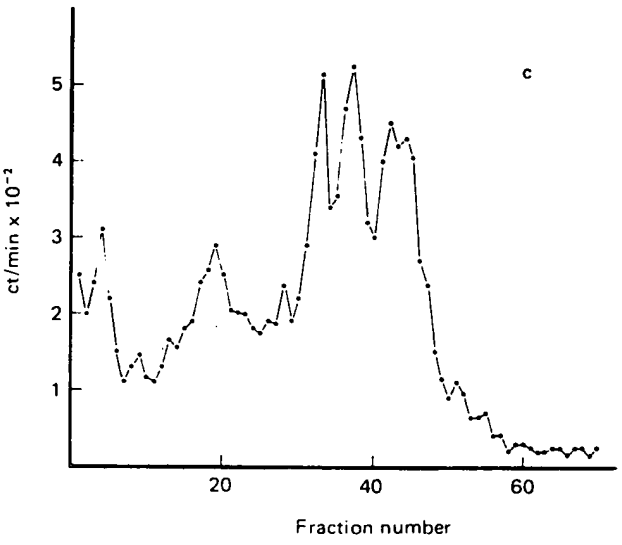


Figure 5 SDS-PAGE analysis of purified IBV grown in CK cell cultures on 7.5% acrylamide gels. (a) Gel stained for polypeptides with Coomassie blue. (b) Gel stained for carbohydrate with Schiff's reagent. (c) [^{3}H]-leucine labelled virus.

The quantitative differences between the polypeptides of IBV grown in eggs or CK cells are not easily explained but may represent differences in host protein incorporation or contamination, the effect of external conditions such as proteolytic enzymes, or genuine differences in the structure of the virus grown in different host systems.

One of the more interesting aspects of coronavirus properties is the increasing evidence suggesting a relationship with the oncornavirus group. Garwes, Pocock and Wijaska (1975) have demonstrated the close similarity between the RNA genomes of two coronaviruses, TGEV and haemagglutinating encephalomyelitis virus of pigs, and Rous sarcoma virus. Collins *et al.* (1976) have observed structures very similar in appearance to the ribonucleo-protein of some oncornaviruses (Bolognesi *et al.*, 1973) in purified preparations of IBV. Representatives of both groups can be shown to possess haemagglutination activity which is only exposed following enzyme treatment involving phospholipase C (Witter *et al.*, 1973; Bingham, Madge and Tyrrell, 1975). The polypeptides of IBV reported in the present study show a remarkable similarity in number, size and glycosylation to those reported for mouse mammary tumour virus (Sarkar and Dion, 1975; Table 1) which is further evidence of a link between the two apparently distinct virus groups.

References

ALEXANDER D.J. 1974. Comparison of the structural polypeptides of four avian paramyxoviruses. *Arch. ges. Virusforsch.* **46** 291–301.

ALEXANDER D.J. and Reeve P. 1972. The proteins of Newcastle disease virus. 1. Structural proteins. *Microbios* **2** 199–212.

ANDERSON N.G., Harris W.W., Barber A.A., Rankin C.J. and Candler E.L. 1966. Separation of subcellular components and viruses by combined rate- and isopycnic zonal centrifugation. *Nat. Cancer Inst. Monogr.* **21** 253–83.

BERRY D.M. and Almeida J.D. 1968. The morphological and biological effects of various antisera on avian infectious bronchitis virus. *J. gen. Virol.* **3** 97–102.

BINGHAM R.W. 1975. The polypeptide composition of avian infectious bronchitis virus. *Archs. Virol.* **49** 207–16.

BINGHAM R.W., Madge M.H. and Tyrrell D.A.J. 1975. Haemagglutination by avian infectious bronchitis virus - a coronavirus. *J. gen. Virol.* **28** 381–90.

BIRNIE G.D., Rickwood D. and Hell A. 1973. Buoyant densities and hydration of nucleic acids, proteins and nucleoprotein complexes in metrizamide *Biochim. Biophys. Acta* **331** 283–94.

BOLOGNESI D.P., Luftig R. and Shaper J.H. 1973. Localization of RNA tumour virus polypeptides. 1. Isolation of further virus substructures. *Virology* **56** 549–64.

BRACEWELL C.D. 1975. Antigenic relationships between strains of infectious bronchitis virus as shown by the plaque reduction test in chicken kidney cells. Proceedings Vth World Congress of the World Veterinary Poultry Association, Munich, 1973.

COLLINS M.S., Alexander D.J. and Harkness J.W. 1976. Heterogeneity of infectious bronchitis virus grown in eggs. *Archs. Virol.* **50** 55–72.

FAIRBANKS G., Steck T.L. and Wallach D.F.H. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry* **10** 2606–17.

GARWES D.J. and Pocock D.H. 1975. The polypeptide structure of transmissable gastroenteritis virus. *J. gen. Virol.* **29** 25–34.

GARWES D.J., Pocock D.H. and Wijaszka T.M. 1975. Identification of heat-dissociable RNA complexes in two porcine coronaviruses. *Nature* **257** 508–10.

HIERHOLZER J.C., Palmer E.L., Whitfield S.G., Kaye H.S. and Dowdle W.R. 1972. Protein composition of coronavirus OC43. *Virology* **48** 516–27.

MOORE N.F. and Burke D.C. 1974. Characterization of the structural proteins of different strains of Newcastle disease virus. *J. gen. Virol.* **25** 275–89.

POCOCK D.H. and Garwes D.J. 1975. The influence of pH on the growth and stability of transmissable gastroenteritis virus *in vitro*. *Archs. Virology* **49** 239–47.

SARKAR N.H. and Dion A.S. 1975. Polypeptides of mouse mammary tumour virus. 1. Characterization of two group-specific antigens *Virology* **64** 471–91.

STEWART M.L., Summers D.F., Soreiro R., Fields B.N., and Maizel J.V. 1973. Purification of oncornaviruses by agglutination with concanavalin A. Proceedings of the National Academy of Sciences of the United States of America. **70** 1308–12.

WATKINS H., Reeve P. and Alexander D.J. 1975. The ribonucleic acid of infectious bronchitis virus. *Archs. Virol.* **47** 279–86.

WITTER R., Frank H., Moënning V., Hunsmann G., Lange J. and Schafer W. 1973. Properties of mouse leukaemia viruses. IV. Haemagglutination assay and characterization of haemagglutinating surface components. *Virology* **54** 330–45.

Accepted 29 December 1976

MICROBIOS

A Prestige International Journal

of Chemical and General Microbiology

PROTEIN EXPRESSION AND PURIFICATION 4, 110–113 (1993)

One-Step Chromatographic Method for the Purification of Avian Serotransferrin

Amandio V. Vieira and Wolfgang J. Schneider[1]

Department of Biochemistry and Lipid and Lipoprotein Research Group, The University of Alberta, Edmonton, Alberta, Canada T6G 2S2, and the Department of Molecular Genetics, Biocenter and University of Vienna, 1030 Vienna, Austria

Received October 20, 1992

Existing purification procedures for serum transferrins involve multistep chromatographic separations and require several days to complete. In addition, they have not been tested for purification of transferrins directly from the blood of egg-laying animals, where large amounts of circulatory lipoproteins can interfere with standard chromatographic separations. We have developed a procedure for purifying transferrin in one step directly from the serum of ovulating chickens. The method, which is based on hydrophobic interaction chromatography, gives a yield of about 12 mg (80%) of purified serotransferrin from 3 ml of serum and can be completed in a few hours. © 1993 Academic Press, Inc.

Purified serum transferrin (Tf) is often used for studies of receptor-mediated endocytosis and as a standard during the characterization of novel cell membrane receptors because its structural properties and cellular uptake mechanism have been well characterized (1–6). Tf is an 80-kDa glycoprotein, produced mainly in the liver, which sequesters iron and transports it into cells via receptor-mediated endocytosis (1,4). More recently, serum Tf has also been identified as a specific regulator of cellular differentiation and proliferation (7–9), a function which appears to be independent of its iron-carrying capacity.

We are currently engaged in studying the transport and uptake of serum nutrient-carrier proteins, such as lipoproteins, vitamin-binding proteins, and Tf, into rapidly growing chicken oocytes (10–12). We are also developing a serum-free medium based on purified chicken proteins and growth factors for studies with transformed and primary chicken cell lines. Our efforts have thus far been limited by the lack of an effective method for obtaining homogeneous preparations of chicken serum Tf.

The reported purification procedures for transferrins from mammalian blood (13–18) incorporate several chromatographic steps and require a few days to complete. There are two reported studies in which chicken serum transferrin was prepared (19,20); both procedures also involve multiple chromatographic steps, and their efficiency and the purity of the resultant Tf has not been reported. We show in this report that a one-step chromatographic procedure based on the differential affinity of proteins for a phenyl-Sepharose matrix column effectively separates chicken Tf from other serum proteins. The method yields milligram quantities of pure Tf from a few milliliters of serum and requires only a small fraction of the time and materials necessary for previously published procedures.

EXPERIMENTAL PROCEDURES

Materials. Phenyl-Sepharose CL-4B and Millex-PF filters (0.8 mm) were obtained from Pharmacia. Conalbumin and horseradish peroxidase-conjugated Protein A were purchased from Sigma. Antibodies used were IgG fractions isolated from immune or nonimmune rabbit serum by chromatography on Protein A Sepharose CL-4B (Pharmacia). Chemiluminescence reagents were obtained from Amersham.

Animals. White Leghorn laying hens (8–18 months old) were obtained from the Department of Animal Sciences, University of Alberta. Adult, female New Zealand white rabbits from a local commercial farm were used for antibody production.

Hydrophobic interaction chromatography. Serum was obtained from White Leghorn laying hens and kept frozen at −20°C. For the purification procedure, a 3-ml

[1] To whom correspondence should be addressed at Department of Molecular Genetics, Biocenter and University of Vienna, Dr. Bohr-Gasse 9/2, A-1030 Vienna, Austria. Fax: (43-1) 79515-2013 or (43-1) 798-6224.

1046-5928/93 $5.00
Copyright © 1993 by Academic Press, Inc.
All rights of reproduction in any form reserved.

serum aliquot was thawed and mixed with 12 ml of buffer A (1.5 M ammonium sulfate, 0.5 M sodium citrate, pH 6). The diluted serum was filtered through a 0.8-μm Millex-PF filter and loaded onto a phenyl-Sepharose column (1.2 cm diameter; 15 ml matrix volume) that had been equilibrated with buffer A. Chromatography was performed at room temperature. After loading the sample, the column was washed with 6 bed vol of buffer A. Elution was begun with buffer B (0.75 M ammonium sulfate, 0.25 M sodium citrate, pH 6) by successive addition of 3-ml vols and collecting the eluate. Protein absorption at 280 nm (A_{280}) was measured for each fraction. Starting with the first protein-containing fraction, 40 μl of each was analyzed by SDS–PAGE. The fractions containing the pure transferrin were pooled, dialized against PBS, and stored at −70°C. Protein was quantitated according to the method of Lowry (21).

The phenyl-Sepharose column was regenerated for subsequent purification procedures by washing with several column volumes of water, 1% SDS, water, ethanol, water, and buffer A, in that order.

Preparation of yolk aqueous extract. Chicken oocytes (1–2 cm follicular diameter) were pierced to obtain yolk and the yolk was frozen at −70°C. In a typical preparation, a 10-ml aliquot of frozen yolk was thawed and mixed with 20 ml of ice-cold H_2O containing 1 mM phenylmethanesulfonyl fluoride and 2 μM leupeptin for 2–4 h at 4°C. The mixture was then spun at 20,000*g* for 25 min. The turbid supernatant was pooled, frozen at −70°C for 2 h, and then thawed at room temperature. The thawed mixture was then recentrifuged (20,000*g*, 25 min). The aqueous bottom phase was collected, filtered through 0.8 μm Millex-PF, and then used for electrophoresis.

Electrophoresis and Western blotting. SDS–PAGE was performed on a 4.5 to 18% polyacrylamide gradient gel according to a standard method (22). For electrophoresis under reducing conditions, the sample was adjusted to 50 mM dithiothreitol. The proteins in the gels were either stained directly with Coomassie blue or transferred to nitrocellulose membranes for Western blotting as previously described (23). The nitrocellulose was blocked with 5% skim milk powder in 50 mM Tris–HCl, pH 7.5, 100 mM NaCl, and subsequently incubated with rabbit IgG. Anti-conalbumin IgG was used at a final concentration of 10 μg/ml and the control, preimmune IgG, was at 7 μg/ml. Bound IgG was detected with horseradish peroxidase–protein A in a chemiluminescence reaction performed as suggested by the supplier (Amersham). Signals on Western blots were quantitated by densitometric scanning on the "Quick Scan" apparatus (Helena Laboratories).

RESULTS AND DISCUSSION

The elution profiles from the phenyl-Sepharose column, as detected by protein absorbance (Fig. 1A) and

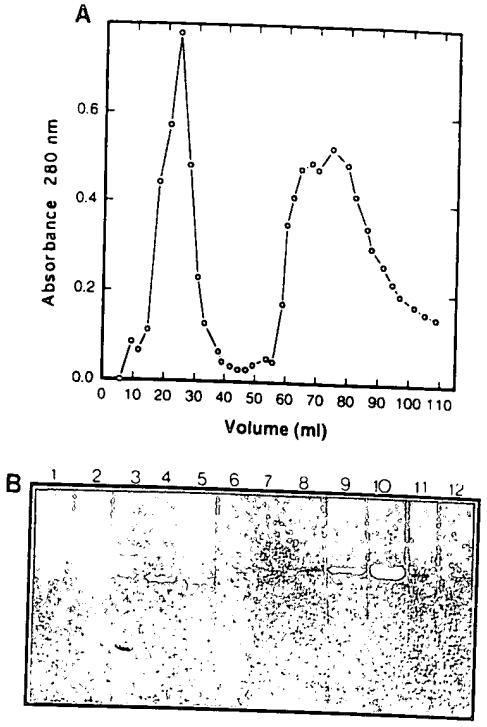


FIG. 1. Elution profiles of chicken serum proteins after hydrophobic interaction chromatography. (A) Fractions were collected and measured for absorbance at 280 nm as described under Experimental Procedures. (B) Selected fractions were separated by SDS–PAGE, 40 μl/lane, and stained with Coomassie blue. The elution volumes (ml) for each fraction (in reverse order of elution) are as follows: lane 1, 101.4; lane 2, 87.4; lane 4, 74.5; lane 5, 67.3; lane 6, 58.7; lane 7, 55.7; lane 8, 37.7; lane 9, 30.7; lane 10, 24.1; lane 11, 14.6; lane 12, 9.4. Lane 3 contains molecular mass standards (kDa): 205, 116, 97, 67, 43, 30.

SDS–PAGE of selected fractions (Fig. 1B), show the early appearance of a 79.4-kDa serum protein. An abundant protein of this size can be seen in the Coomassie-stained gel after electrophoresis of chicken whole serum (Fig. 2A). The relatively high abundance and the molecular weight values of the purified protein suggest it is Tf. Western blots (Fig. 2B) show that the purified protein (lane 2) and a protein with identical mobility in chicken serum (lane 3) and yolk (lane 4) are recognized by an anti-conalbumin polyclonal antibody. Conalbumin, the standard in lane 1 of Fig. 2B, is the egg white form of transferrin which is synthesized by the oviduct of the laying hen. The presence of Tf in the yolk (Fig. 2B, lane 4) has previously been reported (19). Yolk Tf, like most other yolk components which have serum counterparts, is believed to be derived from the uptake of serum Tf during oocyte growth (19,24). By densitometric quantitation, the Tf signal on a Western blot of a series of chicken serum dilutions was compared with the signal of known amounts of the purified protein on the same blot. The serum concentration we obtained was 5 mg/ml for this purified protein (data not shown, cf. Fig. 2B). Starting with 3 ml of chicken serum, we routinely ob-

tained 11–13 mg of the purified protein in the first 30 ml of the Tf-containing column eluate. Thus, the yield of our one-step method is approximately 80%.

The isolated chicken serum protein also shows a large increase in mobility in SDS–PAGE when it is analyzed under nonreducing conditions (Fig. 3). A similar shift is observed for conalbumin (Fig. 3). This shift is a characteristic of the serum Tfs from many other species (25), and has also been detected in a transferrin homologue isolated from the larval haemolymph of the insect *Manduca sexta* (26). Thus, molecular weight, serum levels, antigenic epitopes, and large changes in electrophoretic mobility upon reduction confirm the identity of our purified protein as chicken serotransferrin.

The purity of the Tf preparation was determined by examining the most highly overloaded lane (Fig. 1B, lane 10) which contains 44 μg of protein. Three faint bands (29, 50, and 63 kDa) were seen in this lane on the Coomassie blue-stained gel. Their intensity was lower than the 1-μg bands of the molecular weight standards

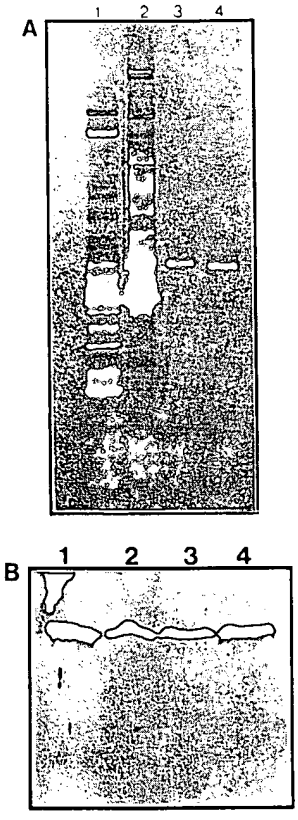


FIG. 2. (A) SDS–PAGE of yolk aqueous extract (5 μl, lane 1), whole laying hen serum (1 μl, lane 2), pure chicken serum Tf (5 μg, lane 3), and conalbumin (6 μg, lane 4). All samples were reduced prior to electrophoresis. (B) Samples identical to those in (A) were electrophoresed on another gel and the proteins were transferred to nitrocellulose. A Western blot was performed with polyclonal antibodies to conalbumin as described under Experimental Procedures: lane 1, conalbumin; lane 2, purified chicken serum Tf; lane 3, laying hen serum; lane 4, yolk extract.

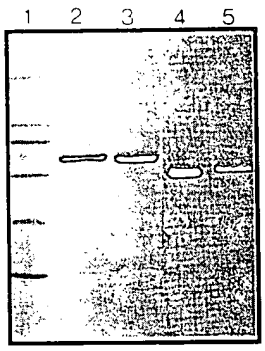


FIG. 3. The effect of disulfide bond reduction on the electrophoretic mobility of purified chicken serum Tf and conalbumin. Both serum Tf (lanes 2, 5) and conalbumin (lanes 3, 4) undergo a large decrease in mobility upon reduction with 50 mM dithiothreitol (lanes 2, 3). Molecular mass standards (kDa) are in lane 1: 205, 116, 97, 67, 43, 30.

of the same gel (data not shown; cf. lane 3, Fig. 1B). Thus, these bands, which may be either contaminants or Tf degradation products, represent at most 1–2% of the total protein.

The fact that the whole 79-kDa Tf band undergoes the large shift under nonreducing conditions to a 63-kDa band (Fig. 3) suggests that there are no contaminants comigrating with the Tf. Hemopexin, for example, which is the common contaminant in previous purification procedures, does not undergo such a shift in mobility (27).

The serum concentrations for various mammalian transferrins have been reported to be in the range of 2–5 mg/ml (14). Our value of 5 mg/ml for chicken transferrin is at the upper level of this range. This high level is in agreement with observations that oviparous animals such as birds have elevated levels of many serum proteins which are destined for the yolk (11,24).

In summary, our results show that Tf, under the high ionic strength conditions used, has a lower affinity for phenyl-Sepharose than most other serum proteins in the ovulating chicken. Based on this difference, we have developed a purification procedure. We speculate that the low affinity for a hydrophobic matrix is due to the inability of the highly disulfide-bridged Tf molecule to unfold and expose the hydrophobic amino acids of its core. The presence of large quantities of circulatory lipoproteins, however, may also be an important contributing factor for the success of this procedure.

ACKNOWLEDGMENTS

These studies were supported by grants from the Medical Research Council of Canada, the Alberta Heritage Foundation for Medical Research, and the Austrian Fonds zur Förderung der wissenschaftlichen Forschung. We thank Calla Shank-Hogue and Rita Lo for excellent technical assistance and Russel Bishop for participating in the early stages of this work.

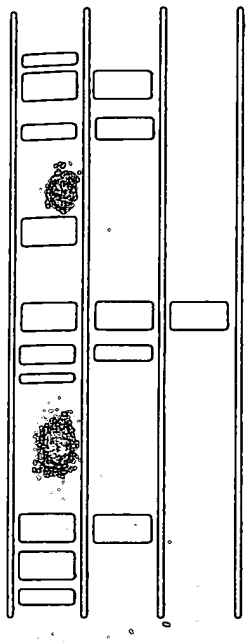
REFERENCES

1. Aisen, P., and Listowski, I. (1980) *Annu. Rev. Biochem.* **49,** 357–393.
2. Divino, C. M., and Schussler, G. C. (1990) *J. Biol. Chem.* **265,** 1425–1429.
3. Lund, K., Opresko, L., Starbuck, C., Walsh, B., and Wiley, S. (1990) *J. Biol. Chem.* **265,** 15713–15723.
4. Newman, R., Schneider, C., Sutherland, R., Voodinelich, L., and Greaves, M. (1982) *Trends Biochem. Sci.* **1,** 397–400.
5. Bretscher, M. S., and Lutter, R. (1988) *EMBO J.* **7,** 4087–4092.
6. Ward, D., and Kaplan, J. (1990) *Biochem. J.* **270,** 369–374.
7. Sibrasku, D. A., Pakala, R., Sato, H., and Eby, J. (1991) *Biochemistry* **30,** 7466–7477.
8. Rossi, M. C., and Bruce, Z. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 6197–6201.
9. Dentsman, S., Hromchak, R., Guan, X., and Bloch, A. (1991) *J. Biol. Chem.* **266,** 14873–14876.
10. Hayashi, K., Nimpf, J., and Schneider, W. J. (1989) *J. Biol. Chem.* **264,** 3131–3139.
11. George, R., Barber, D. L., and Schneider, W. J. (1987) *J. Biol. Chem.* **262,** 16838–16847.
12. Stifani, S., Barber, D. L., Aebersold, R., Steyrer, E., Shen, X., Nimpf, J., and Schneider, W. J. (1991) *J. Biol. Chem.* **266,** 19079–19087.
13. Welch, S., and Skinner, A. (1989) *Comp. Biochem. Physiol. B,* **93,** 417–424.
14. Regoeczi, E., and Hatton, M. (1980) *Am. J. Physiol.* **238,** R306–310.
15. Sawatzki, G., Anselstetter, V., and Kubanek, B. (1981) *Biochem. Biophys. Acta* **667,** 132–138.
16. Eyk, H. G. V., and Leijnse, B. (1968) *Biochem. Biophys. Acta* **160,** 126–128.
17. Cook, N. D., Simpson, R. J., Osterloh, K., and Peters, T. J. (1985) *Anal. Biochem.* **149,** 349–353.
18. Baker, E., Shaw, D. C., and Morgan, E. H. (1968) *Biochemistry* **7,** 1371–1378.
19. Williams, J. (1962) *Biochem. J.* **83,** 355–364.
20. Thibodeau, S. N., Lee, D. C., and Palmiter, R. D. (1978) *J. Biol. Chem.* **253,** 3771–3778.
21. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
22. Laemmli, U. K. (1970) *Nature* **227,** 680–685.
23. Daniel, T. O., Schneider, W. J., Goldstein, J. L., and Brown, M. S. (1983) *J. Biol. Chem.* **258,** 4606–4611.
24. White, H. B., III (1987) *J. Expt. Zool.* **1,** 53–63.
25. Williams, J., Elleman, T., Kingston, B., Wilkins, A., and Kuhn. K. (1982) *Eur. J. Biochem.* **122,** 297–303.
26. Bartfeld, N. S., and Law, J. H. (1990) *J. Biol. Chem.* **265,** 21684–21691.
27. Taketani, S., Kohno, H., and Tokunaga, R. (1987) *J. Biol. Chem.* **262,** 4639–4643.

Volume 4, Number 2
April 1993

PEXPEJ

ISSN 1046-5928



Protein Expression and Purification

EDITOR-IN-CHIEF
Owen W. Griffith

EXECUTIVE EDITORS
Shelby L. Berger
Loran L. Bieber
Richard R. Burgess
John J. Dunn
Max D. Summers
Paula Traktman

ACADEMIC PRESS, INC. *Harcourt Brace & Company*

San Diego New York Boston London Sydney Tokyo Toronto

MAY-18-2000 11:51 LINDA HALL LIBRARYL 816 926 8785 P.03

Vol. 85, pp. 6404–6408, September 1988
Developmental Biology

NOTICE: This material may be protected by copyright law (Title 17, U.S. Code)

A conserved CATTCCT motif is required for skeletal muscle-specific activity of the cardiac troponin T gene promoter

(myogenesis/promoter element/gene transfer)

JANET H. MAR AND CHARLES P. ORDAHL*

Department of Anatomy, University of California, San Francisco, San Francisco, CA 94143

Communicated by Norman H. Horowitz, April 25, 1988

ABSTRACT **Transcription of the cardiac troponin T (cTNT) gene is restricted to cardiac and embryonic skeletal muscle tissue. A DNA segment containing 129 nucleotides upstream from the cTNT transcription initiation site (cTNT-129) directs expression of a heterologous marker gene in transfected embryonic skeletal muscle cells but is inactive in embryonic cardiac or fibroblast cells. By using chimeric promoter constructions, in which distal and proximal segments of cTNT-129 are fused to reciprocal segments of the herpes simplex virus thymidine kinase (HSV tk) gene promoter, the DNA segment responsible for this cell specificity can be localized to the cTNT distal promoter region, located between 50 and 129 nucleotides upstream of the transcription initiation site. The ability of the cTNT distal promoter region to confer skeletal muscle-specific activity upon a heterologous promoter is abolished when it is displaced 60 nucleotides upstream, indicating that its ability to direct skeletal muscle-specific transcription probably requires proximity to other components of the transcription initiation region. Two copies of the heptamer, CATTCCT ("muscle-CAT" or "M-CAT" motif), reside within the 80-nucleotide cTNT distal promoter region. A 3-nucleotide mutation in one of these copies inactivates the cTNT promoter in skeletal muscle cells. Therefore, the M-CAT motif is a distal promoter element required for expression of the cTNT promoter in embryonic skeletal muscle cells. Since the M-CAT motif is found in other contractile protein gene promoters, it may represent one example of a muscle-specific promoter element.**

Recent evidence suggests that activation of a relatively small number of genetic elements is required to initiate the myogenic differentiation program (1–3). A major developmental problem, therefore, is understanding the mechanisms by which the large number of contractile protein genes is activated by such global signals as well as by the subsequent cell lineage- and developmental stage-specific signals that mediate the intricate gene switching patterns accompanying striated muscle development (4). One possibility is that many muscle genes have common cis regulatory elements that account for their coexpression. The presence of short conserved sequence motifs in regulatory regions of many contractile protein genes (5–11) is consistent with such a model but has yet to be directly tested.

We have been using the chicken cardiac troponin T (cTNT) gene as a model to study gene regulatory programs operating during the embryonic development of cardiac and skeletal muscle. The cTNT gene is suited for such an analysis because it is transcriptionally activated, along with a large cohort of other contractile protein genes, at the onset of cardiac and skeletal muscle development (12–15). At late fetal stages, transcription of the cTNT gene is specifically repressed in developing skeletal muscle tissue and strongly up-regulated in cardiac tissue (16). Repression of the cTNT gene does not occur in cultured embryonic skeletal muscle cells unless they are exposed to nerve or nerve extracts (17). Thus, the cTNT gene is subject to complex transcriptional regulation during cardiac and skeletal muscle development.

By using the cTNT transcriptional promoter and upstream region functionally linked to the marker gene chloramphenicol acetyltransferase (CAT; ref. 18), we have shown that efficient expression in embryonic skeletal muscle cells requires only 129 upstream nucleotides (19). Expression in embryonic cardiac cells, however, requires the presence of additional upstream segments (19). In this report, we have analyzed subsegments of the cTNT promoter for the presence of sequence elements capable of governing skeletal muscle-specific transcription. We show that the 80-nucleotide distal region of the cTNT promoter is sufficient to direct skeletal muscle-specific transcription from a heterologous proximal promoter segment. By using site-directed mutagenesis, we further demonstrate that this muscle-specific activity is dependent upon an intact CATTCCT heptamer present in the cTNT distal promoter region. The CATTCCT heptamer is present in the presumptive regulatory regions of other contractile protein genes (8) and may represent one example of a regulatory element shared among muscle-specific genes.

MATERIALS AND METHODS

Enzymes were purchased from New England Biolabs or Boehringer Mannheim and used according to the manufacturers' instructions. Radiolabeled compounds were purchased from Amersham. Protocols for recombinant DNA, restriction endonuclease mapping, and DNA sequence analysis were conducted by standard procedures (20). The plasmid cTNT-129 (ref. 19) contains 129 nucleotides upstream and 38 nucleotides downstream of the cTNT transcription initiation site cloned into the *Hin*dIII site of pBR-CAT (21). cTNT-113 is identical to cTNT-129 except that the segment between position −129 and −114 has been deleted. Chimeric promoters were also constructed in pBR-CAT. The proximal herpes simplex virus thymidine kinase (HSV tk) promoter segment was obtained from the double linker scanner mutant LS-105-95/-56-46 (ref. 22), and the HSV tk distal promoter segment was isolated from pTE2-ΔS/N (ref. 23). The structure of each promoter construction was confirmed by nucleotide sequencing.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: CAT, chloramphenicol acetyltransferase; cTNT, chicken cardiac troponin T; HSV tk, herpes simplex virus thymidine kinase; M-CAT, muscle-CAT heptamer CATTCCT.

*To whom reprint requests should be addressed.

MAY-18-2000 11:52 LINDA HALL LIBRARYL 816 926 8785 P.04

Standard procedures (24, 25) were used for preparation of chicken embryonic muscle and fibroblast cultures. Cells were transfected, harvested, and analyzed for CAT activity as described elsewhere (18, 19, 26, 27). All CAT activity values are expressed in 10^{-2} unit (1 CAT unit = 1 nmol of chloramphenicol acetylated per 30 min at 37°C) per 1×10^6 cells after subtracting the background CAT activity directed by promoterless pBR-CAT in muscle and fibroblast cells (0.34 ± 0.02 and 0.28 ± 0.06, respectively, in unit $\times 10^{-2}$).

RESULTS AND DISCUSSION

The cTNT gene segment between nucleotide positions −129 and +38 (relative to the transcription initiation site; cTNT-129, see Fig. 1*a*) is sufficient to direct expression of a marker gene, CAT, 2 orders of magnitude higher in cultured embryonic skeletal muscle cells than in fibroblast cells (Fig. 2*a*). The high degree of preferential skeletal muscle-specific CAT expression appears to be an inherent property of the cTNT promoter because CAT is equally expressed in skeletal muscle and fibroblast cells under control of the HSV tk promoter (Fig. 2*a*) and other viral and cellular promoters (ref. 19 and data not shown). To begin to characterize the cis element(s) responsible for the skeletal muscle-specific activity of the cTNT promoter, we subdivided cTNT-129 into proximal and distal regions by digestion with the restriction endonuclease *Sma* I, which cleaves between positions −50 and −49. The proximal region contains a nominal TATA motif, transcription initiation site, and part of exon 1, whereas the distal region contains a nominal CCAAT motif and the core recognition sequence for the transcription factor SP-1 (refs. 29−31; see Fig. 1*a*). As expected, neither th proximal nor the distal region of the cTNT promoter was able to direct CAT expression above background levels (Fig. 3).

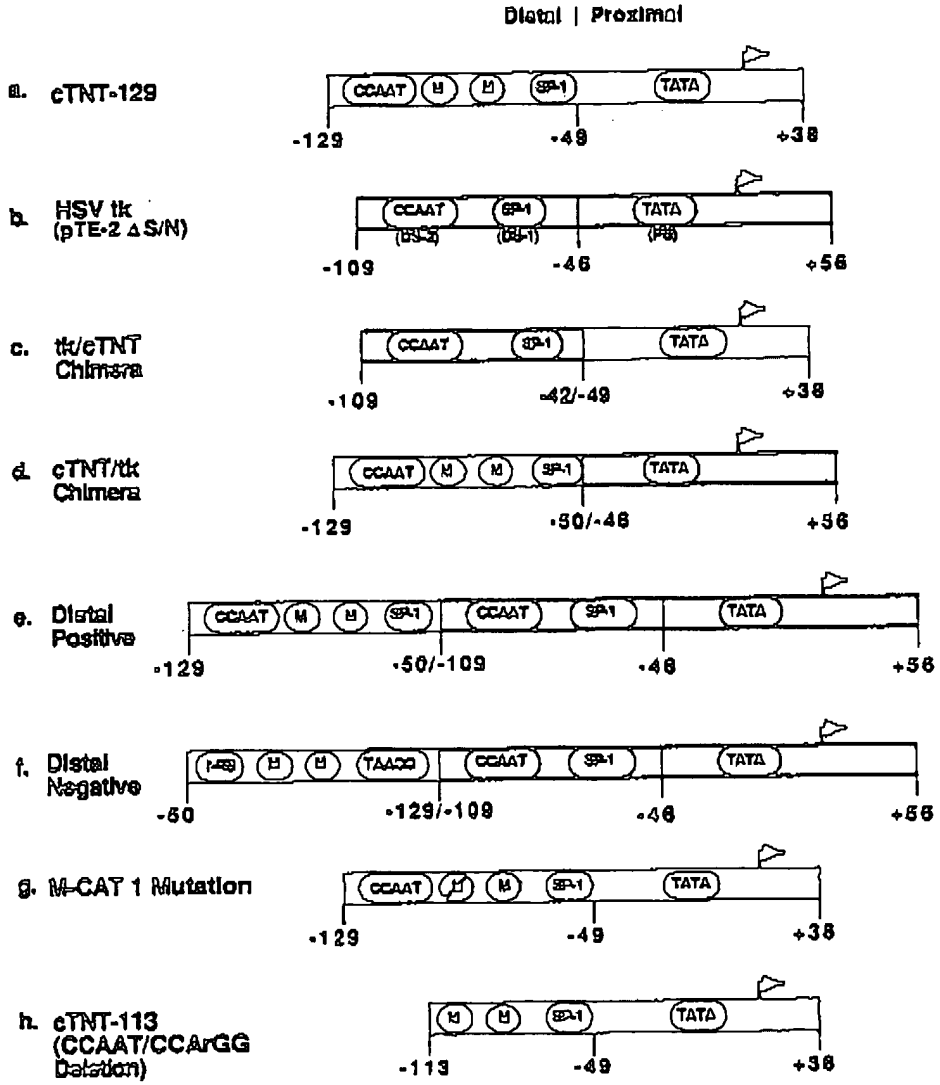


FIG. 1. Structure of cTNT and chimeric cTNT/HSV tk promoters. Diagramatic representation of the promoter constructions used to drive CAT gene expression in these experiments. The proximal and distal regions of *a–d* are indicated. (*a*) The regions distal and proximal to the *Sma* I cleavage site at position −49 are indicated as are the positions of the nominal CCAAT, SP-1, and TATA homologies. M represents the conserved heptanucleotide CATTCCT (see text). (*b*) The plasmid pTE-2ΔS/N carries the HSV tk promoter driving CAT and is described elsewhere (23). The positions of the CCAAT, SP-1, and TATA homologies are shown and correspond to the second distal signal (DS-2), first distal signal (DS-1), and proximal signal (PS), respectively, as defined by McKnight and Kingsbury (28). (*c*) The tk/cTNT chimera contains the tk distal promoter segment fused to the cTNT proximal promoter segment. (*d*) The cTNT/tk chimera contains the cTNT distal promoter segment fused to the HSV tk proximal promoter segment. (*e* and *f*) The distal cTNT promoter segment was inserted into the polylinker region upstream of the tk promoter in either the positive (*e*) or the negative (*f*) orientation. (*g*) The distal conserved heptanucleotide CATTCCT in cTNT-129 was mutated by oligonucleotide-directed mutagenesis. (*h*) BAL-31 deletion of cTNT-129 to position −113 removed the CCAAT/CCArGG homology.

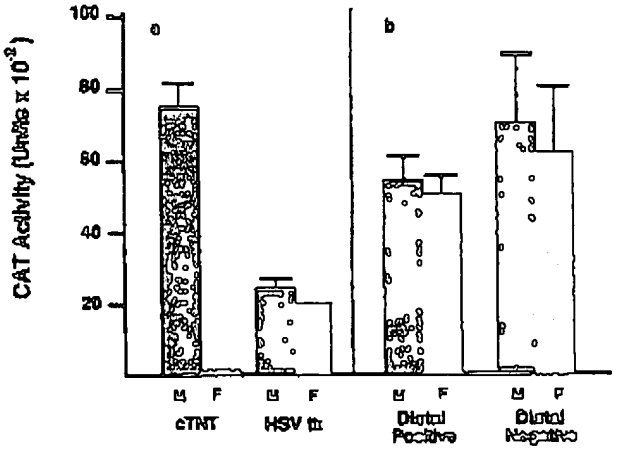


FIG. 2. Promoter activity in muscle and non-muscle cells. (*a*) Comparison of CAT activity under control of the cTNT-129 and the HSV tk promoters (see Fig. 1 *a* and *b*) in skeletal muscle and fibroblast cells. The CAT activity (unit $\times 10^{-2} \pm$ SEM) in fibroblasts (F, solid bars) under the control of cTNT-129 was 0.8 ± 0.04, lower by a factor of 94 than that in skeletal muscle cells (75 ± 6; M, stippled bars). (*b*) Test of the enhancer capability of the cTNT distal promoter segment. Distal Positive and Distal Negative represent plasmids in which the cTNT distal promoter region (see Fig. 1 *e* and *f*) is cloned into the upstream polylinker of pTE-2ΔS/N in either the positive or the negative orientation. Stippled and solid bars indicate activity in skeletal muscle and fibroblast cultures, respectively. Error bars indicate the SEM and are omitted when $<2 \times 10^{-2}$ unit of CAT.

indicating that both a proximal and a distal region are required for cTNT promoter activity.

Construction of Chimeric Promoters. To determine whether one or both regions carry the cis element(s) responsible for restricting transcription to skeletal muscle cells, chimeric promoters were constructed in which the cTNT proximal and distal promoter regions were functionally linked to the reciprocal region derived from the HSV tk gene promoter (see Fig. 1*b*). The HSV tk promoter has been highly characterized as to the functional elements present in its proximal and distal promoter regions, neither of which can function independently (22, 32). The construction of chimeric tk/cTNT and cTNT/tk promoters was made such that the position of the known and putative conserved promoter elements approximated that of the natural promoters (Fig. 1 *c* and *d*).

The chimeric tk/cTNT promoter, containing the tk distal promoter region and the cTNT proximal promoter region, was equally active in skeletal muscle cells and fibroblast cells (Fig. 3), indicating that the cTNT proximal promoter region can functionally complement a heterologous distal promoter region. CAT activity directed by this chimeric promoter is ≈1 order of magnitude lower than that of the parent HSV tk promoter (compare Figs. 2 and 3), which may reflect nonoptimum positioning of various elements within each region relative to one another (22, 33). Alternatively, the cTNT proximal promoter region may, for unknown reasons, function more efficiently with its homologous distal promoter region. In either case, it is clear that the tk/cTNT chimeric promoter supports transcription of the CAT gene at comparable levels in muscle and fibroblast cells. We conclude that the cTNT proximal promoter region does not carry cis elements capable of conferring skeletal muscle transcription to a heterologous distal promoter segment.

In contrast to the above result, the cTNT/tk chimera, in which the cTNT distal promoter region is fused to the tk proximal promoter region, directs CAT expression in muscle cells at a level comparable to that of the tk/cTNT chimera but is inactive in fibroblast cells (Fig. 3). Since the HSV tk

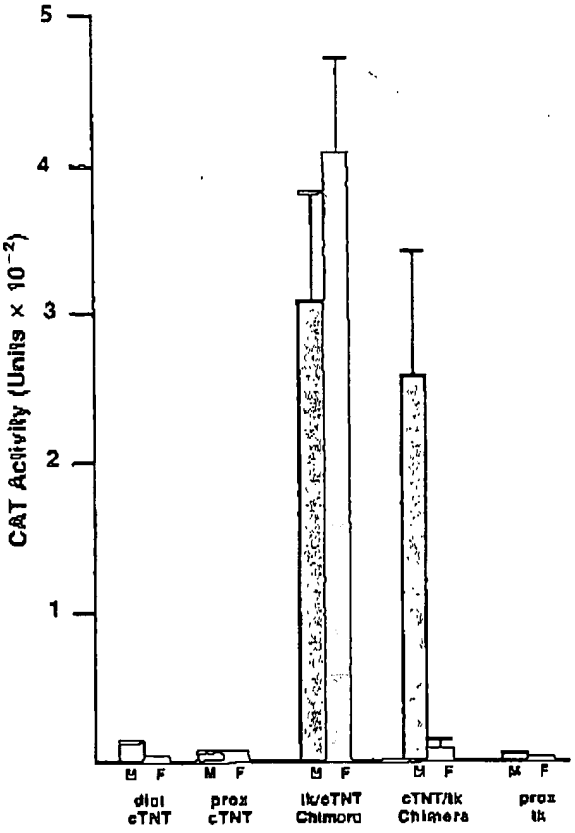


FIG. 3. Skeletal muscle-specific activity of chimeric promoters. Chimeric tk/cTNT and cTNT/tk promoters were tested for their ability to drive CAT expression as described in the legend to Fig. 2. The structures of the tk/cTNT and cTNT/tk chimeras are shown in Fig. 1 *c* and *d*, respectively. In addition, the proximal (prox) and distal (dist) regions of the cTNT and HSV tk promoters were inserted into pBR-CAT and tested for their ability to drive CAT expression independently. M, skeletal muscle cells; F, fibroblasts. Error bars indicate the SEM but are omitted when $<0.02 \times 10^{-2}$ unit of CAT.

promoter carries no skeletal muscle specificity (see above and Fig. 2), and its proximal region is transcriptionally inactive by itself (Fig. 3), the 27-fold higher activity of the cTNT/tk chimeric promoter in skeletal muscle versus fibroblast cells is entirely attributable to the presence of the cTNT distal promoter region. Therefore, the cTNT distal promoter region contains a cis element(s) capable of supporting transcription in skeletal muscle cells but that cannot support, or block, such activity in fibroblast cells.

Enhancer Tests of the cTNT Distal Promoter Region. Since cell-specific transcription of many genes has been shown to be dependent upon the presence of cell-specific enhancer sequences in cis (21, 23, 34–38), we wanted to determine if the distal segment of the cTNT promoter contained a skeletal muscle-specific enhancer. We used position and orientation independence as criteria for enhancer function because prototype enhancers exert their effects over long distances and in positive and negative orientations (39). The cTNT distal promoter segment was inserted in positive and negative orientations into the polylinker cloning site of an enhancer test plasmid, pTE2-ΔS/N, which contains the complete HSV tk promoter driving CAT (see Fig. 1 *e* and *f*; ref. 23). Fig. 2*b* shows that in this upstream position the cTNT distal promoter region elevates HSV tk promoter activity ≈3-fold, in an orientation-independent fashion, as expected for a weak transcriptional enhancer (39). This modest enhancement activity, however, is not skeletal muscle specific because the degree of enhancement is approximately equivalent in skeletal muscle and fibroblast cells (Fig. 2*b*).

MAY-18-2000 11:53 LINDA HALL LIBRARYL 816 926 8785 P.06

Thus, displacement of the cTNT distal promoter region 60 nucleotides upstream completely abolishes its ability to confer preferential expression in skeletal muscle cells. This sharp distance dependence is not expected for a cell-specific enhancer and suggests that the cTNT distal promoter element(s) responsible for its muscle-specific activity may require short-range interaction with other components during transcription initiation.

Muscle-Specific Distal Promoter Motifs. Conserved regulatory sequence motifs have been hypothesized to control coordinate regulation of contractile protein genes. The cTNT distal promoter region contains two sequence motifs that are also found in comparable regions of other muscle-specific genes. One conserved sequence element is centered around the nominal CCAAT homology (Fig. 4*a*; positions −114 to −120). We designated this sequence motif as the nominal CCAAT homology because it resembles the canonical CCAAT homology typical of eukaryotic promoters in its position and sequence, except that it has more adenosine residues than usual (29, 30). Such adenosine-rich CCAAT motifs are a common feature of contractile protein gene promoter regions (see refs. 4, 5, and 10 for examples) and are often embedded within a somewhat longer conserved block dubbed the "CCArGG motif" (where "r" represents repeated adenosine or thymidine residues) by Minty and Kedes (10). In some instances, the CCArGG motif may be unrelated to the canonical CCAAT motif (40).

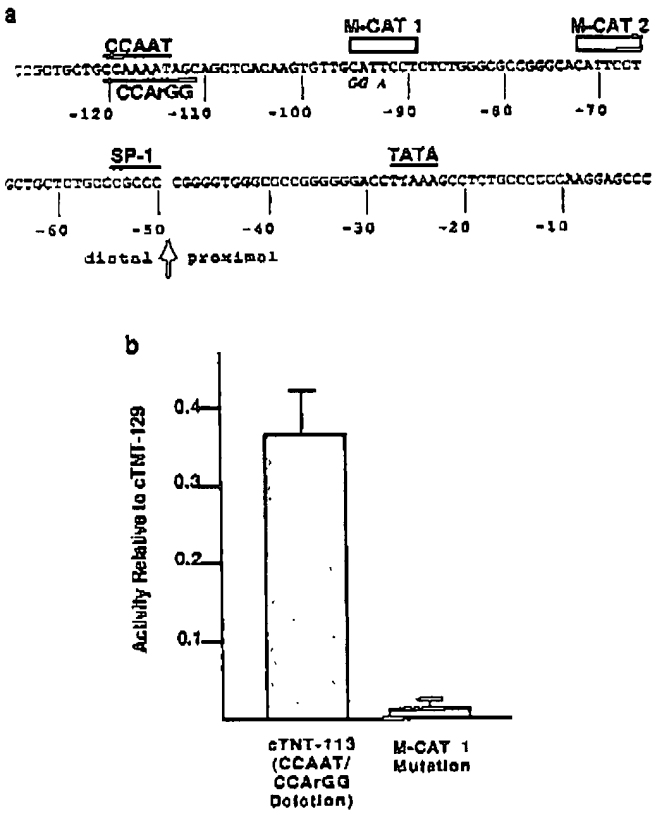


FIG. 4. Effect of mutations within the cTNT distal promoter region. (*a*) Nucleotide sequence of the cTNT promoter region. The sequence of the first 129 nucleotides upstream from the transcription initiation site (15) is shown with the nominal CCAAT, TATA, and SP-1 motifs indicated by overlining. The cleavage site for *Sma* I is indicated by the bold vertical line and separates the cTNT promoter into nominal distal and proximal regions. The locations of the conserved sequence CATTCCT are indicated by the open boxes labeled M-CAT 1 and M-CAT 2. The CCArGG motif (10) is underlined. The nucleotide changes in the M-CAT 1 mutation (positions −95, −94, and −92) are indicated. (*b*) The CAT activity directed by cTNT-129 carrying the M-CAT 1 mutation and that of cTNT-113 (CCAAT/CCArGG Deletion) is plotted relative to that of cTNT-129, which was set at unity. All other details are as described in the legend to Fig. 2.

To test the requirement for the CCAAT/CCArGG motif in the cTNT promoter, the −129/−113 segment was deleted from cTNT-129 to create cTNT-113 (see Fig. 1*h*). The activity of cTNT-113 is approximately one-third that of cTNT-129 in skeletal muscle cells (Fig. 4*b*) but remains inactive in fibroblast cells (data not shown). Therefore, although the CCArGG motif may play an important role in expression of actin genes (11), deletion of the CCAAT/CCArGG motif has only a modest effect upon the activity of the cTNT promoter in skeletal muscle cells. In addition, this result further delimits the cis region primarily responsible for specifying a high degree of skeletal muscle-specific transcription to the 64-nucleotide segment between positions −113 and −50.

Within that 64-nucleotide segment are two copies of another sequence element, CATTCCT, which is found in the promoter regions of many contractile protein genes (8). We refer to the CATTCCT heptamer as the "muscle-CAT" or "M-CAT" motif to distinguish it from the more common CCAAT homology found in many eukaryotic promoters (28, 29). There are two identical copies of the M-CAT motif residing between the nominal CCAAT and SP-1 homologies of the cTNT distal promoter region (M-CAT 1 and M-CAT 2; Fig. 4*a*). The conservation of the sequence and position of M-CAT motifs in some muscle-specific promoters suggests that it may play a role in their muscle-specific regulation.

To test the role of the distal-most CATTCCT motif of the cTNT promoter we changed the first, second, and fourth nucleotides of M-CAT 1 (Fig. 4*a*) by means of oligonucleotide-directed mutagenesis (41) to alter the most highly conserved portion of the heptamer (8). cTNT-129 carrying the M-CAT 1 mutation directed lower CAT activity (by a factor of 50) than wild-type cTNT-129 in skeletal muscle cells (Fig. 4*b*). Moreover, the M-CAT 1 mutation had no effect upon the inability of the cTNT promoter to be expressed in fibroblast cells (data not shown). It is not yet known whether M-CAT 1 requires the presence of an intact M-CAT 2 or CCAAT/CCArGG for full function. However, the fact that a 3-nucleotide change in M-CAT 1 virtually abolishes the activity of the cTNT promoter indicates that, at a minimum, an intact M-CAT 1 motif is essential for cTNT promoter function in embryonic skeletal muscle cells.

Summary and Conclusions. The results presented here demonstrate that the 80-nucleotide distal segment of the cTNT promoter carries a sequence element(s) responsible for directing skeletal muscle-specific transcription. Two sequence motifs within this distal region are shared by other contractile protein gene promoters. One of these, the CCAAT/CCArGG motif, plays an important role in the cardiac actin gene (11) but can be deleted from the cTNT promoter with only a modest effect upon its activity in skeletal muscle cells. In contrast, a 3-nucleotide mutation in a M-CAT motif (CATTCCT) abolishes activity of the cTNT promoter in skeletal muscle cells. Therefore, the M-CAT motif is essential for activity of the cTNT promoter in skeletal muscle cells and may play a similar role in the other muscle promoters in which it is found. It should now be possible to determine the molecular basis for that role.

We thank Steve McKnight and our colleagues at University of California, San Francisco, for making valuable plasmid constructions available. This research was supported by grants to C.P.O. from the National Institutes of Health. J.H.M. was supported by a National Institute of Child Health and Human Development postdoctoral fellowship.

1. Konieczny, S. F. & Emerson, C. P., Jr. (1984) *Cell* 38, 791–800.

2. Konieczny, S. F., Baldwin, A. S. & Emerson, C. P., Jr. (1986) in *Molecular Biology of Muscle Development*, UCLA Symposia on Molecular and Cellular Biology, New Series, eds. Emerson, C., Fischman, D., Nadal-Ginard, B. & Siddiqui, M. A. Q. (Liss, New York), Vol. 29, pp. 21–34.
3. Lassar, A. B., Paterson, B. M. & Weintraub, H. (1986) *Cell* 47, 649–656.
4. Buckingham, M. E. & Minty, A. J. (1983) in *Eucaryotic Genes: Their Structure, Activity and Regulation*, eds. MacClean, N., Gregory, S. P. & Flavell, R. A. (Butterworth, London), pp. 365–395.
5. Ordahl, C. P. & Cooper, T. A. (1983) *Nature (London)* 303, 348–349.
6. Nudel, U., Greenberg, D., Ordahl, C. P., Saxel, O., Neuman, S. & Yaffe, D. (1985) *Proc. Natl. Acad. Sci. USA* 82, 3106–3109.
7. Chang, K. S., Rothblum, K. N. & Schwartz, R. J. (1985) *Nucleic Acids Res.* 13, 1223–1237.
8. Nikovits, W., Jr., Kuncio, G. & Ordahl, C. P. (1986) *Nucleic Acids Res.* 14, 3377–3390.
9. Bergsma, D. J., Grichnik, J. M., Gossett, L. M. A. & Schwartz, R. J. (1986) *Mol. Cell. Biol.* 6, 2462–2475.
10. Minty, A. & Kedes, L. (1986) *Mol. Cell. Biol.* 6, 2125–2136.
11. Miwa, T. & Kedes, L. (1987) *Mol. Cell. Biol.* 7, 2803–2813.
12. Ordahl, C. P., Kioussis, D., Tilghman, S. M., Ovitt, C. & Fornwald, J. A. (1980) *Proc. Natl. Acad. Sci. USA* 77, 4519–4523.
13. Ordahl, C. P., Cooper, T. A., Ovitt, C., Fornwald, J. A., Calman, A. F. & Caplan, A. I. (1982) in *Muscle Development: Molecular and Cellular Control*, eds. Pearson, M. L. & Epstein, H. F. (Cold Spring Harbor Lab., Cold Spring Harbor, NY), pp. 225–236.
14. Cooper, T. A. & Ordahl, C. P. (1984) *Science* 226, 979–982.
15. Cooper, T. A. & Ordahl, C. P. (1985) *J. Biol. Chem.* 260, 11140–11148.
16. Long, C. S. & Ordahl, C. P. (1988) *Dev. Biol.* 127, 228–234.
17. Toyota, N. & Shimada, Y. (1983) *Cell* 33, 297–304.
18. Gorman, C. M., Moffitt, L. F. & Howard, B. H. (1982) *Mol. Cell. Biol.* 2, 1044–1051.
19. Mar, J. H., Antin, P. B., Cooper, T. A. & Ordahl, C. P. (1988) *J. Cell Biol.* 107, in press.
20. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
21. Walker, M. D., Edlund, J., Boulet, A. M. & Rutter, W. J. (1983) *Nature (London)* 306, 557–561.
22. McKnight, S. L. (1982) *Cell* 31, 355–365.
23. Edlund, T., Walker, M. D., Barr, P. J. & Rutter, W. J. (1985) *Science* 230, 912–916.
24. Konigsberg, I. R. (1978) in *Methods in Enzymology*, eds. Kakoby, W. B. & Pastan, I. (Academic, New York), Vol. 58, pp. 511–527.
25. Rubin, H. (1957) *Virology* 4, 533–562.
26. Graham, F. & van der Eb, A. (1973) *Virology* 52, 456–467.
27. Gorman, C., Merlino, G. T., Willingham, M. L., Pastan, I. & Howard, B. H. (1982) *Proc. Natl. Acad. Sci. USA* 79, 6777–6781.
28. McKnight, S. L. & Kingsbury, R. (1982) *Science* 217, 316–325.
29. Breathnach, R. & Chambon, P. A. (1981) *Annu. Rev. Biochem.* 50, 349–383.
30. Benoist, C. O., Hare, K., Breathnach, R. & Chambon, R. (1980) *Nucleic Acids Res.* 8, 127–142.
31. Kadonaga, J. T., Jones, K. A. & Tjian, R. (1986) *Trends Biochem. Sci.* 11, 20–23.
32. McKnight, S. L., Gavis, E. R., Kingsbury, R. & Axel, R. (1981) *Cell* 25, 385–398.
33. Cochran, M. D. & Weissmann, C. (1984) *EMBO J.* 3, 2453–2459.
34. Mason, J. O., Williams, G. T. & Neuberger, M. S. (1985) *Cell* 41, 479–487.
35. Grosschedl, R. & Baltimore, D. (1985) *Cell* 41, 885–897.
36. Hammer, R. E., Krumlauf, R., Camper, S. A., Brinster, R. L. & Tilghman, S. M. (1987) *Science* 235, 53–58.
37. Choi, O.-R. & Engel, J. D. (1986) *Nature (London)* 323, 731–734.
38. Emerson, B. M., Nickol, J. M., Jackson, P. D. & Felsenfeld, G. (1987) *Proc. Natl. Acad. Sci. USA* 84, 4786–4790.
39. Khoury, G. & Gruss, P. (1983) *Cell* 33, 313–314.
40. Miwa, T., Boxer, L. M. & Kedes, L. (1987) *Proc. Natl. Acad. Sci. USA* 84, 6702–6706.
41. Lewis, E. D., Chen, S., Kumar, A., Blanck, G., Pollack, R. E. & Manley, J. L. (1983) *Proc. Natl. Acad. Sci. USA* 80, 7065–7069.

LEVEL 1 - 6 OF 6 REFERENCES

National Library of Medicine MEDLINE Database

TITL: Transgenic birds from transformed primordial germ cells.

AUTH: Vick L; Li Y; Simkiss K

ORGA: Department of Pure and Applied Zoology, School of Animal and Microbial Sciences, University of Reading, U.K.

CITE: Proc R Soc Lond B Biol Sci 1993 Mar 22; 251 (1332): 179-82

LANG: ENG; English

ABST: Primordial germ cells (PGCS) are the progenitor cells for the gametes. They can be obtained from avian embryos by dissociating the germinal crescent region, where they accumulate, or by sampling the blood at the time of their migration to the gonad. PGCS were obtained from these sources and transfected with defective retroviruses. These manipulated cells were injected into recipient embryos to form chimaeras which grew to sexual maturity and produced offspring, some of which contained the foreign DNA. This is the first example of the direct use of PGCS to produce transgenic offspring. (AUTHOR)

MJTR: Animals, Transgenic. Chickens GE.

MNTR: Animal. Blotting, Southern. Chick Embryo. Chimera. Defective Viruses GE. DNA GE. DNA IP. Female. Germ Cells PH. Male.

AUG.21'98 13:42 FR CISTI URGENT SERVICE TO 17033053585 P.03/34

Applicants copy

4

Transgenic birds

K. SIMKISS

4.1 Introduction

Birds are virtually unique among domesticated species in being bred for the nutrient value of their ova, and few organisms have been subjected to as much genetic selection as poultry. These two features encapsulate both the attractions and the frustrations of producing transgenic birds.

Current breeding practices in the poultry industry depend on natural genetic variation that is exploited by selective reproduction. New variants are introduced from mutations or translocations, but there is a progressive loss of 'wild-type' characteristics. By inserting foreign DNA into organisms it is possible to bypass reproductive barriers and induce gene flow between vastly different organisms from quite distinct populations. Thus, the potential benefits of genetic engineering are extremely attractive, for they could dramatically increase both the genetic repertoire and the rate of change of a particular breed. Unfortunately, one of the characteristics of birds that makes them so commercially attractive – their large eggs – is also one of the features that makes it necessary to devise new approaches to their genetic engineering. The most common way of introducing foreign DNA into mammals is microinjection into the male pronucleus (Palmiter & Brinster 1986). The large size of birds' eggs makes it virtually impossible to see these structures and, to make matters worse, polyspermy is common. There may be up to 20 male pronuclei in a fertilized fowl egg with no indication as to which will eventually fuse with the female pronucleus. For these reasons microinjection is difficult unless special methods are used, and as a result most approaches to avian transgenesis have used other techniques, based mainly upon gaining access to

AUG. 27 '98 13:42 FR CISTI URGENT SERVICE TO 17033053585 P.04 34

the avian genome via a variety of embryonic cells. Fortunately, the bird's embryo is probably the best-studied example of vertebrate development, and this has been of great benefit to these approaches. Unfortunately, other aspects of avian biology are not as well documented. Avian karyotypes are characterized by a few megachromosomes and a large number of microchromosomes. The first attempts have been made at producing a genetic map of the fowl (Bumstead & Palyga 1992), and it is estimated that there are between 50,000 and 100,000 genes present (Shuman 1990). Only about one-thousandth of these have been cloned, however, so that a great deal of the potential value of genetic engineering remains unidentified (Bulfield 1990).

It will be apparent, therefore, that it has not been possible simply to adapt the technology of mammalian transgenesis to birds. Instead, new methods have had to be devised, often with considerable ingenuity. The subject is now at the stage where progress is being made and where many of the results from previous studies of avian embryology and biology can be capitalized upon with all the potential benefits of disease resistance, metabolic manipulation and enhanced understanding.

4.2 Avian reproduction

4.2.1 Gametes

4.2.1.1 Spermatozoa

Birds do not possess accessory reproductive organs that produce seminal fluid. There is, however, a lymphlike fluid generated during the erection of the phallic structures in the cloaca that is referred to as 'transparent fluid'. The role of this fluid during ejaculation is not clear, but fertilization can occur in its absence. Such semen contains about 6×10^8 spermatozoa per cubic millimeter (Lake 1971). A number of attempts have been made to use the sperm as a vehicle for transferring adventitious genetic material into the germ-line. The simplest approach to this technique was that described by Pandey and Patchell (1982). Sperm were disabled by radiation and then used to inseminate hens of a different strain. After 24 hr the hens were inseminated with fresh sperm from cocks of their own strain. The theory was that the irradiated sperm would be carried into the ovum by the active sperm, and it was claimed on the basis of feather and egg colour markers that this occurred in between 3 and 5% of cases. These results have been

AUG.21'98 10:43 FR CISTI URGENT SERVICE TO 17033053585 P.05

confirmed by Tomita *et al.* (1988) and by Bumstead *et al.* (1987), who used this method to introduce the major histocompatibility haplotype marker into progeny. Work by Shoffner *et al.* (1987), however, failed to reveal any evidence for the transfer of marker genes from irradiated sperm, and it is not entirely clear what gene insertion mechanism is actually envisaged as occurring with this approach. Even more controversial results followed a report by Lavitrano *et al.* (1989) that the sperm of mice could be coated with strands of foreign DNA, which were then carried into the ovum on fertilization. A number of attempts were made to repeat this experiment, but Brinster *et al.* (1989) were unable to produce any transgenic mice by such sperm-mediated DNA transfer despite analyses of 1,300 mice produced in this way. Similar detailed attempts to transfer linearized plasmid (5.5-kb pCK17 or 2.7-kb pUC19) using fowl sperm showed that from 350 to 4,000 plasmid modules attached to each sperm but that none of this was incorporated into the progeny (Gavora *et al.* 1991). This result confirmed the observation that foreign DNA is rapidly attached to the head of sperm but that there is no evidence for the production of transgenic birds from such gametes. Abstracts are still being published claiming that between 30 and 60% of offspring from DNA-coated rooster sperm contain foreign DNA that is transmitted into the F1 generation (Gruenbaum *et al.* 1991), but doubt must be expressed about these results until the full experimental details are published.

The idea of using sperm as a vector to transfer exogenous DNA into the ovum is so attractive that liposomes have also been used to introduce genes into sperm. This technique was used successfully to incorporate foreign DNA into mouse sperm, but no transgenic offspring were produced (Bachiller *et al.* 1991). This approach has not been tried in birds.

4.2.1.2 Ova

Most birds have only one functional ovary, usually the left one, which lies deep in the body cavity. Relatively few attempts have been made to insert genes directly into the developing follicle, although Shuman (1986) used replication-competent reticuloendotheliosis virus (REV) for that purpose. Fertilized eggs from such birds were incubated and virus was found in 26% of cases. Encouraged by these results, Shuman repeated the experiments using a replication-deficient retrovirus, and 8% of the offspring showed signs of this DNA.

AUG.21'98 13:43 FR CISTI URGENT SERVICE TO 17033053585 P.06/34

Similar experiments were undertaken by Kopchick *et al.* (1991), who used surgery to puncture the air sacs and expose the avian follicles. These were injected with 1–25 μg of a plasmid (pBGH). Of the 400 offspring that were eventually produced, none was found to possess the foreign DNA, and it was suggested that the inserted DNA was rapidly degraded in the oocyte.

Because of the surgical procedures that are necessary and the relatively poor results that were obtained, few attempts have been made to use the ovary as a site for DNA insertion. It is possible to fertilize avian oocytes *in vitro,* and avian sperm does not require capacitation in the female tract, so that this process is relatively easy to perform (Howarth 1971). Unfortunately, however, the avian oocyte rapidly degenerates during the 24 hr it takes from the time it is ovulated to the time it is laid. It is therefore not possible to attempt either fertilization or genetic engineering on an infertile newly laid egg, and consequently the prospects for using the female gamete in such studies are virtually nonexistent.

4.2.2 The embryo

The embryology of the domestic fowl is very well described for both normal and pathological states (Lillie 1919; Romanoff 1972). The requirements of genetic manipulation, however, have directed attention to two relatively neglected aspects. These are, first, the earliest stages of cell proliferation and differentiation that occur before egg laying and, second, the development of the germ-cell lineage. The interest in these stages relates directly to the possibility of using either embryonic stem (ES) cells or primordial germ-cells (PGCs) as vehicles for producing transgenic birds by producing germ-line chimeras.

4.2.2.1 Development before oviposition

About 2 hr before the oocyte is released from the ovary of the fowl, it undergoes its first reduction division to form a secondary oocyte and the first polar body. Sperm, introduced into the oviduct at copulation, are partly stored in special glands at the uterovaginal junction. Some, however, rapidly pass up the reproductive tract so that fertilization occurs in the infundibulum region almost immediately after ovulation. Polyspermy is common, with an average of about 10 sperm entering the small region of yolk-free cytoplasm that forms the germinal disc.

AUG.21'98 13:43 FR CISTI URGENT SERVICE TO 17033053585 P.07

Here they lose their cytoplasm and swell to about 50 times their volume to form the male pronuclei (Perry 1987). Shortly after this, the second maturation division of the oocyte occurs to form another polar body and the female pronucleus. Fertilization, in the sense of the fusion of the male and female pronuclei, occurs shortly afterwards, and the supernumerary male pronuclei slowly degenerate. The fertilized egg passes down the oviduct and is covered in a number of tertiary 'membranes' secreted by the surrounding epithelium and its glands. The magnum region secretes the albumen, and the egg passes through this region in about 2.5 hr to reach the isthmus, or shell-membrane-forming region. At this stage it has probably completed its first cell division, and it then enters the pouchlike shell gland at the distal end of the oviduct, where it is 'plumped' by watery secretions until it is roughly twice its original size. During this process, which takes about 18 hr, the egg is rotated and the eggshell is secreted. Cell division continues throughout this period, so that when the egg is laid it consists of about 60,000 cells, of which about half are destined to be extraembryonic (Spratt & Haas 1960). Four aspects of this phenomenon are important. The first is that the albumen is converted from a high Na/K secretion of about 10:1 to a product with an Na/K ratio of about 2:1 (Mongin & Sauveur 1970). Second, the rotation of the egg at a rate of about 1 revolution per 4 min twists the albumen to form the chalazae. Third, the effect of gravity during this process determines the anterioposterior axis of the future embryo. Finally, the subembryonic fluid develops, separating an overlying cellular layer, or area pellucida, from the underlying yolk (Eyal-Giladi 1984).

Understanding these events and their relationship to both embryo culture and the establishment of cell commitments is crucial to many of the approaches involved in producing transgenic birds (Simkiss 1993). This has been greatly facilitated by the description of 14 stages in the initial cleavage and development of the blastoderm. These are identified and described as I to XIV by Eyal-Giladi and Kochav (1976). The main components of this analysis are the recognition of three developmental periods starting with cleavage (stages I–VI), which takes about 10 hr and results in the entire germinal disc being converted to a uniform epithelium, the blastoderm. The second period involves the formation of the area pellucida (stages VII–X), in which an upper layer of smaller cells separates from a lower layer of larger cells. This area pellucida appears as a transparent zone that becomes

AUG.21'98 13:44 FR CISTI URGENT SERVICE TO 17033053585 P.08/34

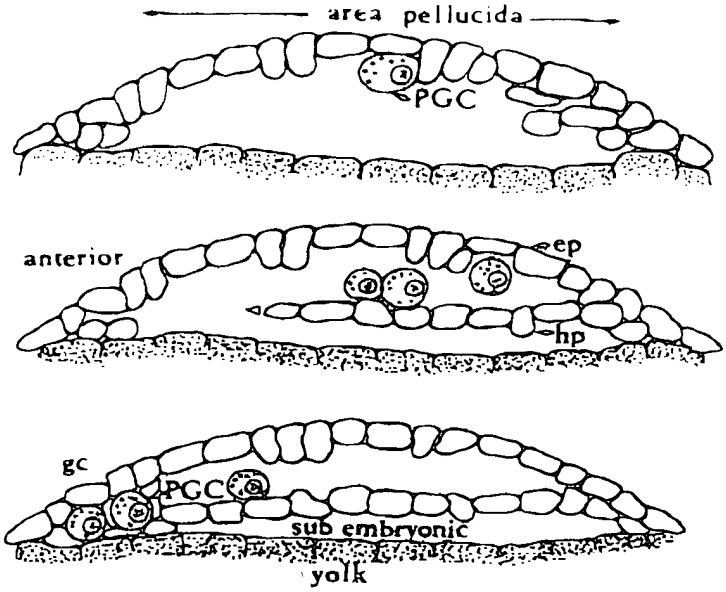


Fig. 4.1. Diagrammatic vertical sections of the blastoderm at stage X (top), stage XII (middle) and stage 6 (bottom). Anterior to left. The initial uniform blastoderm consists of a transparent area pellucida overlying a subembryonic fluid and an opaque area opaca (top). By stage XII this layer has separated into an upper epiblast (ep) and a forming hypoblast (hp, middle). Primordial germ-cells (PGCs), originating from the epiblast, are carried anteriorly on the hypoblast and accumulate at the germinal crescent (gc, bottom).

progressively more clearly demarcated from the surrounding area opaca due to the formation of the subembryonic fluid between the blastoderm and the underlying yolk. This stage of development has normally been reached by the time the egg is laid, which in descriptive embryology is considered day 0 (i.e., no incubation). It is important to realize, however, that cell division has been occurring and the blastoderm has been developing for between 20 and 24 hr by this stage. The period of hypoblast formation (stages XI–XIV) involves the separation of the blastoderm into two layers, an upper epiblast and a lower hypoblast. The process of gastrulation involves the subsequent inward migration of cells from the epiblast to form the notochord and mesoderm of the embryo. A simplified composite drawing of this system and the way the primordial germ-cells are involved over the period of the first 2 days of incubation is shown in Fig. 4.1. It should be realized, however, that there is still some controversy over the details of the involvement of the posterior section of the area pellucida in hypoblast formation (Stern 1990; Eyal-Giladi *et al.* 1992).

AUG.21'98 13:44 FR CISTI URGENT SERVICE TO 17033053585 P.09/34

4.2.2.2 Development during incubation

The initial events in embryo development have already been outlined, since they extend over stages XI–XIV of the Eyal-Giladi and Kochav scheme. This period, however, is also covered by the classification of Hamburger and Hamilton (1951), which starts with the initiation of incubation and progresses through primitive streak formation (stage 2, 6–7 hr) and the formation of the first somite (stage 7, 23–6 hr incubation) to the formation of the lung primordia (stage 15, 50–5 hr), appearance of the allantois (stage 18, 65–9 hr) and eventual hatching (stage 46, 480–504 hr). Stages XI–XIV of Eyal-Giladi and Kochav overlap stages 1–2 of the Hamburger and Hamilton classification. Much of the interest in the Hamburger and Hamilton scheme is associated with organogenesis. For the purpose of this review, this involves the formation of the gonads and, by implication, the separation of the germ-line.

In all vertebrates, the cells that will form the gametes arise at sites some distance away from the developing gonads. The explanation for this is not clear, but it may be a strategy to protect the germ-line from the consequences of differentiation in the somatic cells. One of the results of this extragonadal origin of these PGCs, however, is that they must migrate, during embryogenesis, to find and populate the gonadal stroma (Nieuwkoop & Satasurya 1979). This implies some complex cellular signalling between the migrating cell line (PGCs) and the target organ (gonadal stroma) with all its implications for gene activation and expression. In the birds the PGCs have been identified as arising from the blastoderm of freshly laid eggs at stage X (Muniesa & Dominguez 1990) and leaving the epiblast at stages XII–XIII (Ginsburg & Eyal-Giladi, 1987) to be carried anteriorly on the hypoblast and mesodermal edges to a position to the anterior of the embryo, the germinal crescent (see Fig. 4.1). Thus, by the first day of incubation (stages 4–8) they occupy a position that is not only extragonadal but actually extraembryonic. In the duck, Fargeix (1969) counted between 50 and 100 PGCs in the germinal crescent of stage 2–5 embryos, and by stages 10–15 this had increased to between 200 and 400. In the quail, it is possible to use monoclonal antibodies to detect a few isolated cells in the unincubated blastoderm (stage X), with about 90 PGCs at stages 2–3 and several hundred by stages 8–9 (Pardanaud *et al.* 1987a). Similar numbers were found in the embryo of the fowl by Al-Thani and Simkiss (1991) until around stage 12 of incubation, when, as in other birds, they begin to migrate from this site into the vascular

AUG 21 '98 13:44 FR CISTI URGENT SERVICE TO 17033053585 P.10/34

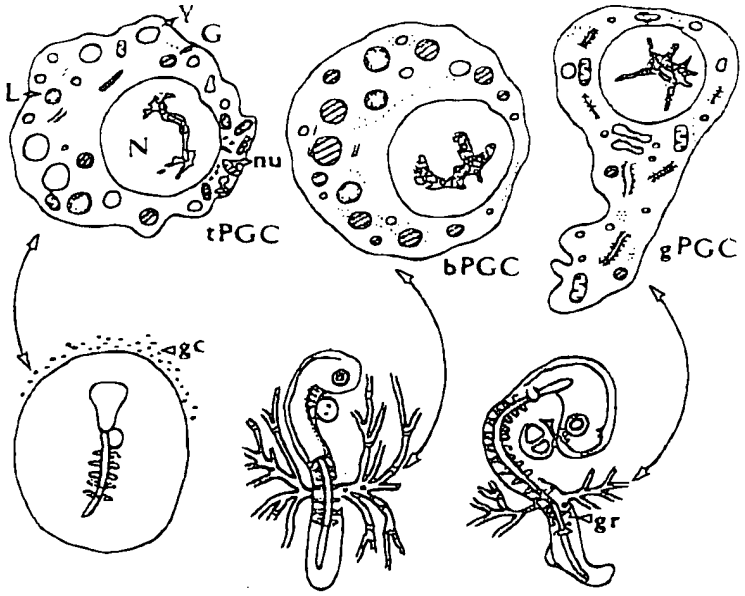


Fig. 4.2. Primordial germ-cells (tPGC) from the germinal crescent region of a stage 12 embryo (left), from the vascular system (bPGC) of a stage 16 embryo (centre) and from the germinal ridge (gPGC) of a stage 18 embryo (right). L, lipid droplet; Y, yolk granule; G, glycogen deposit; N, nucleus; gc, germinal crescent; gr, germinal ridge, nu, nuage. (After Simkiss, 1991.)

system. The PGCs appear to be capable of some active movement at this time, but their incorporation into the circulatory system is largely dependent on the formation of a vascular network and the onset of cardiac activity. A population of migrating PGCs in the blood was first identified by Meyer (1964) at about stage 16 of development; shortly after this stage, the germ cells can be found in increasing numbers in the gonadal anlagen (Fig. 4.2). The PGCs are attracted to the gonad by chemical signals (Kuwana *et al.* 1986), but their exit from the circulation may be facilitated by the constriction of the blood vessels in this region. Once the PGCs have entered the gonadal anlagen, they divide rapidly to produce more than half a million oocytes in the fowl. This number then crashes, so that only a few thousand persist in the 4-day-old hatchling (Hughes 1963).

It will be apparent from this brief description that it is possible to isolate the cells that will form the gametes from (a) presumptive blastoderm (pPGC), (b) germinal crescent tissue (tPGC), (c) circulating blood (bPGC) or (d) gonadal sources (gPGC). In the case of (a) it is possible to culture pieces of blastoderm to produce PGCs (Ginsburg & Eyal-Giladi 1989), while in the cases of (b), (c) and (d) it is possible to

AUG.21'98 13:45 FR CISTI URGENT SERVICE TO 17033053585 P.11

harvest a considerable number of these cells from embryos. Such cells provide an opportunity for the insertion of foreign DNA into the germ-line if such genetically manipulated cells are subsequently injected into the recipient embryo to form a germ-line chimera. This possibility was explored by Simkiss *et al.* (1989), who isolated PGCs from the blood of a stage 15 embryo with a DNA marker and injected them into a recipient embryo without this DNA. The resulting chimera had a gonad containing germ-cells from both embryo sources. In pursuing this approach it should be recognized that the different developmental stages of PGCs (pPGC, tPGC, bPGC and gPGC) not only are morphologically distinct (see Fig. 4.2) but also have a variety of membrane receptors and cellular properties that may be very important for producing transgenics.

4.3 Approaches to avian transgenesis

4.3.1 Microinjection and embryo culture

As already mentioned, the injection of foreign DNA into the pronucleus of the avian egg suffers from two difficulties. The first is the problem of identifying this structure in the cytoplasm of an extremely large and opaque cell. The second is determining which of the supernumerary pronuclei in the germinal disc will be involved in the syngamic fusion. Neither of these problems has been resolved, but a number of interesting experiments have been performed. Cloned DNA was injected into the germinal disc, about 25 μm below the plasma membrane in the region of the female pronucleus of a freshly fertilized egg. In *Xenopus* such experiments have resulted in a low frequency of incorporation of the foreign DNA into the host genome (Etkin & Pearman 1987). In similar experiments with fowl eggs using a bacterial gene encoding chloramphenicol acetyltransferase under the control of the Rous sarcoma virus promoter (pRSV cat), the DNA appeared to remain episomal. The linearized plasmid replicated up to 20-fold in the first 24 hr, but the rate then declined and by day 7 was restricted to the extraembryonic membranes (Sang & Perry 1989). In similar experiments using *lac Z* as a reporter gene, histochemical methods could be used to demonstrate β-galactosidase expression (Naito *et al.* 1991a). In this case a chicken *β-actin–lac Z* hybrid gene *(MiwZ)* was used and tissue expression remained in 40% of the embryo at day 4 of

AUG 21 '98 13:45 FR CISTI URGENT SERVICE TO 17033053585 P.12/14

incubation. It was suggested that some exogenous DNA may have integrated into the genome of these cells in a mosaic fashion. In order to pursue this possibility, Perry *et al.* (1991) examined the fate of injected DNA in greater detail by studying the temporal and spatial expression of the *lac Z* gene under the control of the cytomegalovirus promoter (pHFB GCM). Analysis of the DNA in the 26 hr blastoderm showed that in 80% of the embryos only 10% or less of the injected DNA remained, while in the other 20% the whole injected dose was recovered. Maximum expression of the reporter gene occurred around stage X, and normal cell death and dilution by cell division were probably the major influences in its subsequent decline. By day 7 only 8% of the surviving embryos contained any cells expressing the foreign gene.

Experiments which involve the microinjection of the freshly fertilized ovum require eggs that have not passed down the oviduct. This usually means killing the bird to obtain this material and culturing the ovum in a surrogate egg (Rowlett & Simkiss 1985; Rowlett 1991). Devising such a culture system has been difficult, involving several changes of fluid (Perry 1988), the removal of the albumen capsule (Naito *et al.* 1990), the provision of an eggshell as a source of embryonic calcium (Ono & Wakasugi 1984; Rowlett & Simkiss 1987) and rocking the cultures (Deeming *et al.* 1987). One reason for these difficulties is the fact that under normal circumstances the albumen that is secreted by the bird has an Na/K ratio that is extensively modified during its passage down the oviduct (Section 4.2.2.1) and it is difficult to simulate this in a static system (Simkiss *et al.* 1993; Simkiss 1993). These culture problems have been largely overcome by removing much of the original albumen, and it is now possible to obtain hatchability rates of almost 50% (Naito *et al.* 1990). The procedures, however, are difficult and time-consuming and add to the problems of using microinjection of the pronucleus to produce transgenic birds. For these reasons most experimenters have subsequently attempted to introduce foreign DNA into partially incubated eggs.

4.3.2 The blastoderm

By far the most successful way of producing transgenic birds to date has been to infect the blastoderm with either competent or replication-defective retroviruses.

AUG.21'98 13:46 FR CISTI URGENT SERVICE TO 17033053585 P.13

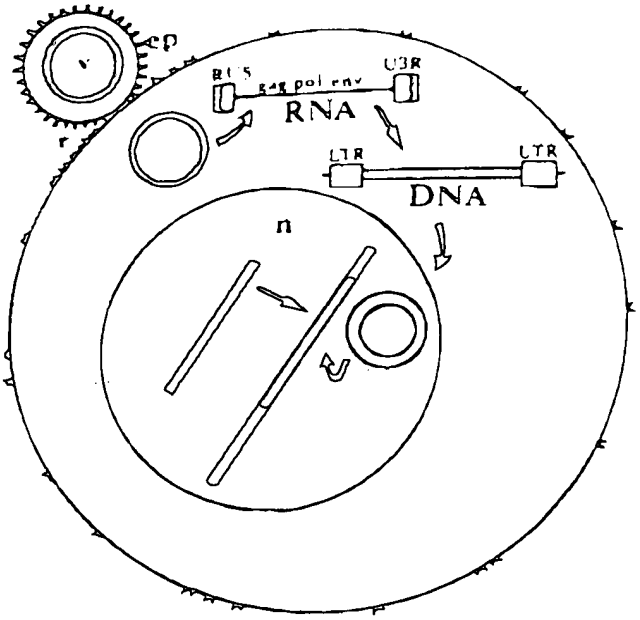


Fig. 4.3. A virion (v) attaches by its envelope proteins (ep) to receptor (r) molecules on the cell surface and inserts its double-stranded RNA into the cytoplasm, where it is transcribed into a double-stranded DNA. This enters the nucleus (n) as a circular structure that inserts as a provirus into the host genome. The retrovirus terminal sequences (RV5, U3R) are replaced by the long terminal repeats that are essential for the transcription of the *gag* (antigen), *pol* (polymerase) and *env* (envelope) genes.

4.3.2.1 Retroviruses

Retroviruses are RNA viruses that replicate through a region of chromosomally integrated proviral DNA (Fig. 4.3). The viral sequences include the trans-acting regions that encode viral proteins and the cis-acting components involved in replication. The trans-acting region of the provirus contains three protein coding regions, *gag*, *pol* and *env*. The *gag* gene is involved with viral encapsidation, *pol* encodes for reverse transcriptase, protease and integrase enzymes, while *env* produces the viral envelope. The cis-acting regions include the promoter sequences, the termination signal, the primer binding site and the packaging signal (Shuman 1991). The retrovirus enters specific cells by binding to a membrane receptor protein that is recognized by the viral envelope. Upon entering the cell, the RNA genome is released and transcribed into a linear DNA form. This is transported to the nucleus, where it is ligated into a circular form that integrates into the host chromosome as a provirus. During subsequent transcription and translational activity, new viral particles are produced through the

AUG 21 '98 13:46 FR CISTI URGENT SERVICE TO 17033053585 P.14/34

host cell pathways. These viral components form new virions that bud from the cell membrane.

After entering the cell the retrovirus usually blocks the surface receptor so that only one copy of the viral genes is present in any one cell (Varmus 1988). Provirus integration can occur at multiple sites, but these are probably the more transcriptionally active regions and highly preferred sites have been discovered in the genome (Shih *et al.* 1988).

4.3.2.2 Retroviral transgenics

The first transgenic birds were produced by injecting avian leukosis virus (ALV) into the blastoderm region of newly laid eggs from a retrovirus-free strain of White Leghorns (so-called line zero birds). The eggs were sealed after the injection and incubated normally. Twenty-one males were raised to sexual maturity and shown to contain the retrovirally derived DNA in their sperm. There were 23 different proviral inserts in these birds, which were stably inherited for at least two generations (Salter *et al.* 1986, 1987).

Subsequent studies revealed some interesting aspects of this approach. Of the 23 proviral inserts produced in such experiments, 21 coded for the complete ALV but 2 were incomplete. One coded for only the envelope protein while the other also included a group-specific antigen that induced resistance to these subgroup A viruses (Crittenden & Salter 1990). Since these 2 retrovirus inserts (*alv*6 and *alv*13) were genetically incomplete, they were not infective and only parts of the viral genome were transmitted with that of the bird. They were, however, able to express the genes for the viral coat protein and thus were resistant to subsequent infection of the bird by the same virus type. Processes similar to the type detected in these experiments are presumably responsible for the occurrence of endogenous viral *(ev)* regions in the normal avian genome. By these processes replication-competent retroviruses generate, by chance deletions, transgenic birds that are capable of expressing some remnants of the original viral genome but without the ability to infect other birds. Male birds do not transmit ALV congenitally to their progeny (Spencer *et al.* 1980), so that any evidence for the transfer of such genetic material is interpreted as germ-line transmission.

A replication-competent strain of RSV was constructed by Chen *et al.* (1990) that contained the bovine growth hormone gene *(bGH)*.

AUG 21 '98 13:46 FR CIST URGENT SERVICE TO 17033053585 P.15 34

Approximately 10^5 virus particles were injected into the blastoderm to produce viraemic chicks that, on maturation, produced hens that laid infected eggs in roughly 50% of cases (Kopchick *et al.* 1991). Male offspring from this generation were tested for their ability to transmit proviral sequences. Among those cocks that were able to do this, the transmission rate was between 0 and 4%, indicating that not all of the germ-cells had been infected and that integration had occurred at different sites. Subsequent breeding experiments showed that stable germ-line integration of the exogenous retroviral DNA had occurred, but Southern blots for *bGH* demonstrated that this sequence had been lost. With a second construct the reverse transcriptase gene was replaced and *bGH* was linked to murine metallothionein promoter. Infected blastoderms produced hatchlings containing the construct but with poor correlations in serum *bGH* levels. It appeared that many of the cells secreting the growth hormone were in a variety of somatic tissues.

4.3.2.3 Replication-defective vectors

Replication-defective retroviruses are so called because they are missing either trans-acting or cis- and trans-acting regions (Shuman 1991). As a result they are unable to complete more than one round of the life cycle and cannot produce infectious particles. They are therefore produced in specially constructed helper cells that provide the deleted genes necessary for virus production (Watanabe & Temin 1983). By inserting foreign genes that are driven from either long terminal repeats (LTRs) or internal promoters, it is possible to use replication-defective viruses to introduce and express a variety of foreign genes in the host cell.

A replication-defective spleen necrosis virus (SNV) was used by Bosselman *et al.* (1989a,b) to introduce two marker genes into the blastoderm of fowl eggs. The resulting 760 offspring showed evidence of vector-derived DNA in 23% of cases, and four cocks were obtained that contained these sequences in their sperm. All four birds produced transgenic offspring representing 34 different sites of integration. In a subsequent experiment the chicken growth hormone gene (*cGH*) was introduced into the vector. Embryos injected with this vector did not hatch well, but thirty 15-day-old embryos were analysed for serum growth hormone levels, and in 16 cases these were at least 10 times the level of normal controls (Bosselman *et al.* 1990).

Germ-line transmission of a replication-defective SNV vector has

AUG.21'98 13:47 FR CISTI URGENT SERVICE TO 17033053585 P.16/34

also been obtained in quail. Of 16 male birds produced by blastoderm injection, only one showed germ-line transmission, although this was stable over five generations and expressed the genes in a wide variety of tissues (Lee & Shuman 1990).

4.3.3 Embryonic stem cells

The inner cell mass of the preimplantation mouse embryo contains pluripotent cells which, if injected into other embryos, produce viable chimeras (Gardner & Papaioannou 1975). Culturing such cells was extremely difficult, however, until Evans and Kaufman (1981) induced delayed implantation of these embryos, which then showed a retarded development. Cells obtained from the inner cell mass of these isolated blastocysts could be maintained in culture by using feeder cells. Permanent cell lines were subsequently established from these ES cells, and have been used as cellular vectors for experimental manipulation of the mouse germ-line (Thomas & Capecchi 1987). A number of techniques have been developed using differentiation inhabitant factor to retain these ES cells in a pluripotent state suitable for use in germ-line chimeras (Nichols *et al.* 1990).

The normal preimplantation inner cell mass of the mouse blastocyst contains relatively few cells, and it is not clear what the equivalent stage is in an avian embryo. In reviewing the literature on this subject, Eyal-Giladi (1984) concluded that the stage X blastoderm of the newly laid egg had a concealed bilateral symmetry but that individual cells were pluripotent and epiblast cells may even be totipotent. Evidence to support this derives from the attempts of Marzullo (1970) to produce chick chimeras by mixing blastoderm cells from White Leghorn and Barred Plymouth Rock to Rhode Island Red embryos. It was demonstrated that the transferred cells contributed to feather pigmentation, although no viable chicks were produced. Petitte *et al.* (1990) repeated and extended these observations. Of 53 dwarf White Leghorns injected with Barred Plymouth Rock blastoderm cells, 6 were chimeric for feather colour and 1 male survived to hatching. Mating this bird produced 719 chicks, of which 2 indicated that some germ-line incorporation of cells had occurred; this was confirmed by DNA fingerprinting. This degree of chimerism is low compared with that obtained in mice (Robertson 1986), which raises concern about how pluripotent such stage X blastoderm cells actually are. This question was pursued by Naito *et al.* (1991b), who transferred quail blastoderm cells from a newly laid egg into the stage X blastoderm of the fowl. With an

AUG 21'98 13:47 FR CISTI URGENT SERVICE TO 17033053585 P.17 34

injection volume of 3–5 μl only 38 out of 441 embryos survived, but when the volume was reduced to 1 μl, 48 out of 199 chicks hatched. A total of 7 chimeric chicks were produced, warranting further analysis of such birds. The experiments were repeated by injecting between 700 and 2,000 quail cells into the centre of state XI–XIII blastoderms or into the centre or posterior of stage XIV blastoderms (primitive streak stage) of the fowl. The embryos were killed at 6 days incubation. Histological analysis showed that ectodermal chimerism was limited to the head region when injections were made into the centre of stage XI–XIII blastoderms. Ectodermal and mesodermal chimerism occurred in both types of stage XIV injection. In all three experimental conditions, quail PGCs were found in the embryos and 16 out of 26 individuals (i.e., 62%) were germ-line chimeras. It is not clear from this experiment whether this was due to the random incorporation and differentiation of pluripotent cells or to the selective incorporation of cells that were already developmentally committed.

The use of ES cells for producing transgenic birds has two attractions. The first is that by isolating such cells it will be possible to use nonretroviral vectors to introduce foreign DNA into them. The second is that the ES cells will be capable of being cultured and selected *in vitro* before being used to produce chimeras. Chicken blastoderm cells have been transfected with a variety of plasmids, including heat shock or metallothionein promoters and *lac Z* genes (Brazolot *et al.* 1991). Transfection rates of up to 1 in 25 cells were obtained depending on the quantity of Lipofectin used and the subsequent identification by X gal staining of the enzyme β-galactosidase. Injecting such blastoderm cells into 58 stage X embryos produced 36 specimens at 65 hr in which gene activity was detected, although it was mainly extraembryonic. In one case, however, β-galactosidase-expressing cells were found in the brain, head and ventricle. There is good reason to believe that blastoderm cells can be transfected by a variety of standard techniques to produce chimeras, but the second requirement, of producing a stem cell that is pluripotent for the germ-line and capable of being maintained in cell culture, remains the main obstacle to this approach.

4.3.4 Primordial germ-cells

There is an extensive literature on the transfer of a variety of cells between embryos to form chimeras (Le Douarin & McLaren 1984). In

Fig.
cally
but s

som
dev
indi
circ
Rey
fow
the
of
use
chi
ba:
inf
va
qu
ba
PC
Re
br
ge
PC

AUG 21'98 13:47 FR CISTI URGENT SERVICE TO 17033053585 P.18/34

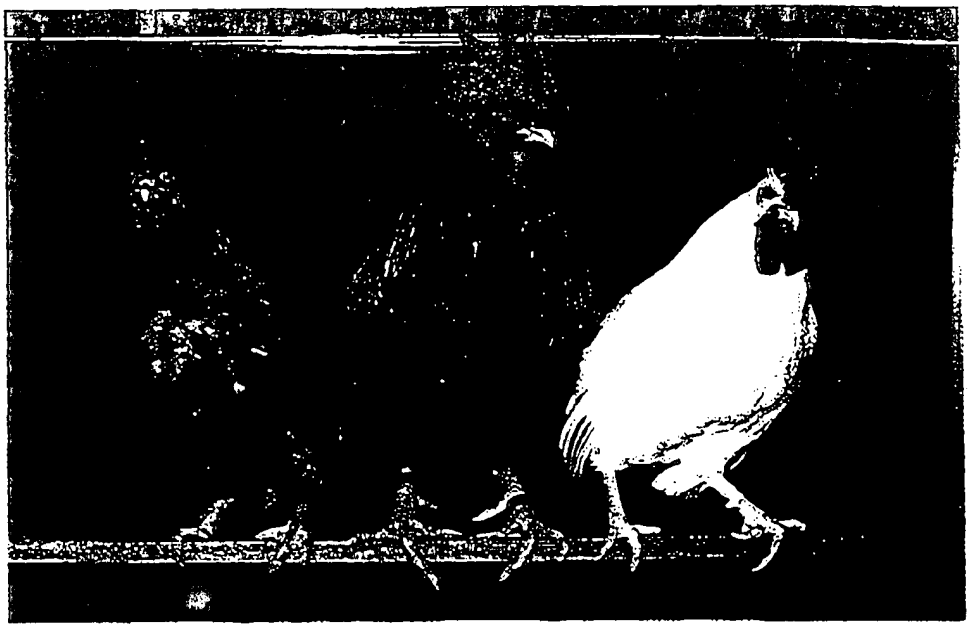


Fig. 4.4. The chimeric Rhode Island Red cockerel (centre) contains genetically manipulated White Leghorn PGCs. Most of its offspring are brown (left), but some have the phenotype of the White Leghorn and are transgenic (right).

some elegant experiments using embryos in culture, Simon (1960) devised a variety of parabiosis experiments to introduce PGCs into individual chick embryos and also to exchange them between the circulations of duck and fowl embryos. In an extension of this work, Reynaud (1969) used intravascular injections of PBCs to create both fowl and turkey–fowl chimeras (Reynaud 1976). The difficulty in all these experiments, however, was to establish the unequivocal survival of the transferred PGCs, and for this reason Simkiss *et al.* (1989) used specific DNA markers to demonstrate the formation of germ-line chimeras in the fowl. Subsequently, a replication-defective retrovirus, based on SNV, was prepared (Meyers *et al.* 1991) and shown to infect fowl PGCs. These manipulated bPGCs were injected into the vasculature of 3-day-old embryonic fowl and specific SNV DNA sequences were recovered from the gonads. Using a defective retrovirus based on a defective avian leukosis virus, Vick *et al.* (1993) transfected PGCs of the White Leghorn and transferred them to a Rhode Island Red embryo. Cocks produced in this way produced some normal brown offspring and some white transgenics, clearly derived from the genetically manipulated PGCs (Fig. 4.4). It is clear, therefore, that PGCs can be used as vehicles for introducing foreign DNA into the

AUG 21'98 13:48 FR CISTI URGENT SERVICE TO 17033053585 P.19

genome of the bird, and subsequent experiments have been directed at characterizing this phenomenon and increasing its efficiency.

The protocol for producing transgenics by such a procedure would, in outline, consist of (a) isolating PGCs, (b) integrating foreign DNA into their genome, (c) screening the cells for suitable gene expression, (d) introducing these genetically manipulated cells into a recipient embryo to form a chimera and (e) breeding from this chimera and selecting those offspring that contained the introduced genes.

4.3.4.1 Isolating primordial germ-cells

There are three well-characterized sources of PGCs in the embryo. These are the germinal crescent tissue (tPGC), blood-borne PGCs (bPGCs) and gonad-derived cells (gPGCs). These correspond to the three progressive stages in the origin, migration and settlement of these cells, and as such they might be expected to show different physiological properties. Morphologically, the germinal crescent PGCs are roundish in shape, with large numbers of yolk granules, ribosomes and mitochondria (see Fig. 4.2). Once they enter the vascular system they develop a few microvilli, and bPGCs typically have a large eccentric nucleus with increasing numbers of glycogen granules in the cytoplasm. On entering the germinal ridge and becoming gPGCs, they often become elongated (up to 20 μm) with numerous cytoplasmic projections and microvilli (Ukeshima & Fujimoto 1984). These features can be associated with increased motility, a conversion of cytoplasmic reserves to glycogen and an increase in protein metabolism. Corresponding changes occur in the membrane proteins. The lectin concanavalin A binds to glucose- and mannose-containing receptors, and it shows a strong affinity for the PGC membrane (Lee *et al.* 1978). In its presence the normal migration of PGCs becomes inhibited (Al-Thani & Simkiss 1991). A number of other lectins (Yoshinaga *et al.* 1992), monoclonal antibodies, such as EMA-1 (Urven *et al.* 1988), and carbohydrate antigens (Loveless *et al.* 1990) are associated with PGC membranes at various stages of this maturation process. This suggests that the membrane receptors on PGCs undergo considerable changes during their migration, and confirmation of this has come from studies on mammals.

In the mouse, the dominant-white spotting (W) and steel (S1) loci have been known for many years to be involved in the migration

AUG 21 '98 13:48 FR CISTI URGENT SERVICE TO 17033053585 P.20 34

of PGCs. It is now apparent that these loci are responsible for a transmembrane tyrosine kinase receptor (stem cell receptor) on the surface of the PGC and a secretion (stem cell factor) from cells associated with the migration path (Witte 1990; Motro *et al.* 1991). The stimulation of such membrane receptors is probably also responsible for inducing the proliferation and differentiation of PGCs and accounts for the fact that migratory and postmigratory PGCs behave quite differently (Donovan *et al.* 1986). It appears that a wide range of these growth factors, including basic fibroblast growth factor (bFGF) and soluble leukaemia inhibitory factor (sLIF), influence the PGC during this period (Resnick *et al.* 1992). It might be expected, therefore, that attempts to produce chimeras by transferring tPGCs, bPGCs and gPGCs into recipient embryos would have different effects. There is evidence that both tPGCs and bPGCs can be transferred from donor to recipient embryos and settle in the germinal ridge. These results come from histological examination of both intra- (Simon 1960) and interspecific (Reynaud 1969) transfers and from DNA analyses of recipient embryos (Simkiss *et al.* 1989; Savva *et al.* 1991). Gonocytes released from avian gonads after the PGCs have settled are of three types, which can be grown in large numbers in culture (Wentworth & Wentworth 1991). These cells appear, therefore, to be released from their mitotic block, but the question as to whether they can be reintroduced into embryos to form chimeric gonads is, as yet, unresolved. Long-term culture of PGCs, to form what Resnick *et al.* (1992) call embryonic germ-cells, produced the speculation that these would rarely, if ever, colonize the germ-line (McLaren 1992), but this does in fact appear to be possible in mice (Stewart *et al.* 1994).

4.3.4.2 Introducing foreign DNA

To date the main method of introducing foreign DNA directly into PGCs has involved the use of normal or replication-defective retroviruses (Simkiss *et al.* 1990). There is no inherent reason why electroporation, calcium phosphate precipitation, DEAE-dextran or Lipofectin liposomes should not be used (Wentworth *et al.* 1992), except that during the development of procedures using PGCs it is most convenient to use vectors that are likely to give the highest rates of integration and at the present time these are retroviruses.

AUG 21 '98 13:48 FR CISTI URGENT SERVICE TO 17033053585 P.21/34

RECEIVED
MAY 25 2000
TECH CENTER 1600/2900

4.3.4.3 Screening PGCs

One of the central attractions of using ES cells in producing transgenics is that they can be genetically manipulated, maintained in long-term culture and then screened for gene expression before being used to produce germ-line chimeras. In this they are superior to PGCs, which at present can be maintained in culture for only short periods and proliferate very poorly (Donovan *et al.* 1986). A number of extracellular matrix components have been tested to determine their influence on avian PGC growth, but they appear to have little effect until 7 days after application (Bellin *et al.* 1985; Wentworth 1989). Attention has therefore shifted to the effects of feeder cells and in particular a number of growth factors (SCF, FGF, LIF; see Section 4.3.4.1). These may represent the normal factors that stimulate the rapid proliferation that occurs with PGCs once they have settled in the germinal ridge. The results of such experiments are very encouraging (Resnick *et al.* 1992) and indicate that it may soon be possible to maintain and screen PGCs in culture.

4.3.4.4 PGC transfer and proliferation

The expression and stimulation of a variety of membrane receptors on PGCs appear to be an essential part of their development, migration to the germinal ridge and subsequent proliferation in the gonad. It is not clear what the temporal and spatial relationships are in these interactions. The system appears, however, to be fairly robust in that a large number of workers have shown, histologically, that both tPGCs and circulating bPGCs can be transferred to recipient embryos and shown to develop in the host germinal ridge (Section 4.3.4). Indeed, several groups have shown that PGCs from mice will populate the germinal ridge of chick embryos (Rogulska *et al.* 1971; Ferhane-Tachinante 1975). It is therefore rather surprising that Petitte *et al.* (1991) were unable to detect any offspring among 3,177 chicks derived from dwarf White Leghorn PGCs that had been injected into 59 Barred Plymouth Rock embryos. These results suggest either that the PGC transfers were not effective for technical reasons (age of embryos, too few PGCs or poor injections) or that there is considerable sorting of PGC cells within the stroma of the developing gonad. In a study of the ovary of the fowl, Hughes (1963, 1964) found that the number of germ-cells rose to 27,000 on day 9 of incubation, reached 680,000 on day 17

AUG 21 '98 13:49 FR CISTI URGENT SERVICE TO 17033053585 P.22/34

and then crashed to only a few thousand in the 4-day-old chick. The fate of individual cells and their relationship to the original 400 gPGC clones that form in the fowl gonad is not known. Clearly, any discrimination in cell sorting in the gonad would have major implications for the production of transgenics by any procedure that involves these chimeras.

4.4 Perspectives

The location of the gonads deep in the body cavity and the size and constitution of the oocytes, with a long delay in oviposition, mean that both the anatomy and physiology of the bird present serious obstacles to the microinjection of DNA into the egg. Thus, unless there is some major technological advance involving, for example, sperm coating with DNA or a pronucleus-seeking vector, it is unlikely that avian transgenics will be produced from the zygote. There are therefore only two viable targets for avian genetic engineering: the blastoderm *in situ* and the embryo chimera. In both cases this means that transgenics will not be produced until the F1 generation, and then they will occur in only a proportion of the offspring. Clearly, if this approach is to be used in the future, technological efficiency will be a major concern.

4.4.1 Manipulating the blastoderm in situ

Manipulating the blastoderm *in situ* has been both the most popular and most successful way of introducing foreign DNA into the avian genome. Most of the transgenics that have been produced have been obtained by direct infection of blastoderm cells *in ovo* using either competent (Section 4.3.2.2) or replication-defective retroviruses (Section 4.3.2.3). The advantages of this approach are (a) technical simplicity of the injection and (b) good success rate. The disadvantages are (a) the use of retroviruses that are difficult to construct and that have a number of inherent problems (size of DNA insert, stability, resistance to use in food products, etc.) (Temin 1989) and (b) somatic as well as germ-line insertion. Of the pros and cons of this approach the use of retroviruses dominates the discussion. An attempt has been made to use cationic liposomes to introduce the RSV LTR and the firefly luciferase gene (*pRSVL*) into the blastoderm (Rosenblum & Chen 1991). High luciferase activity was found in 3-day-old embryos with detectable activity in 8-day-old embryos, but the results again

AUG 21'98 13:51 FR CISTI URGENT SERVICE TO 17033035585 P.23/34

suggested an episomal rather than an integrated fate for the foreign DNA.

4.4.2 *Embryo chimeras*

There are two approaches to producing chimeras by isolating, manipulating and then reintroducing cells into embryos in the hope that they might be involved in forming the germ-line. In their monograph on germ-cells in chordates, Nieuwkoop and Satasurya (1979) wrote, "Although we have a reasonable understanding of the early development of the avian embryo we know nothing about the actual mode of origin of the PGCs in this group." Two types of experiment have shed a light on this problem in the past 24 years. The first have indicated that a specific gene product (*oskar*) may determine the number of PGCs in *Drosophila*. The suggestion is that this gene product may be identified as granules in the cytoplasm of germ-cells (i.e., nuage) and that this material may protect the germ-line from somatic differentiation signals (Ephrussi & Lehman 1992). If the same system operates in chordates, we clearly need to know which cells contain this cytoplasmic factor (or nuage or germ-plasm) and what effect it has on foreign DNA insertion and expression. In the second set of experiments, electron microscopists and immunohistochemists have claimed to have identified PGCs in stage X blastoderms – that is, before incubation (Pardanaud *et al.* 1987b) and before they descend onto the hypoblast (see Fig. 4.1). To make the matter even more significant, at least two groups of workers have claimed that stage 3–4 avian PGCs contain nuage (Climent *et al.* 1979; Muniesa & Dominguez 1990). Those workers who are looking for ES cells have taken the stage X blastoderm as their starting material. It appears at least possible that in doing this they have actually selected a group of cells which include PGCs or precursors that are already committed to forming them in the next few hours. By breaking up the blastoderm, they have partially delayed this process. According to Ginsburg and Eyal-Giladi (1989), "In cultures of stage X blastoderms the morphologic expression of PGCs is related to the level of differentiation and organization of the somatic cells in the culture which, in turn, is dependent on the initial concentration of cells in the culture". It is therefore worth considering that experiments described as using 'stage X embryonic stem' and 'stage 10 primordial germ cell' may be dealing with almost the same stages of the same cell line.

AUG 21'98 13:51 FR CISTI URGENT SERVICE TO 17033053585 P.24 34

4.4.2.1 Plasmid insertion and cell screening

The attraction of using isolated cell lines is that it is possible to use reporter genes to identify and select those cells that have successfully integrated and expressed the foreign DNA. Thus, the relative inefficiency of inserting plasmid DNA into embryonic cells can be overcome by forming chimeras only from those cells that survive the screening process. Blastoderm cells have been transfected with a number of plasmids using the liposome Lipofectin (Brazolot *et al.* 1990). Transfection rates of up to 5% were obtained, and these cells were then injected into recipient embryos. The reporter gene was, however, *lac Z* so that, although it was possible to trace the transfected cells in the embryo, no attempt was made to select these cells before forming a chimera. By contrast, Page *et al.* (1991) used a plasmid vector containing the *neo* gene to transfect embryo fibroblasts again using Lipofectin. The *neo* gene confers resistance to the amino glycoside antibiotic G418, which normally kills these fibroblasts within 2 weeks. Transformed cells were maintained in culture for 2 months in the presence of this antibiotic but not reintroduced into the embryo.

Thus, the complete experiment, on which the stem cell concept is based, has not yet been undertaken in birds. Embryonic cells have not, to date, been transfected, screened and then used to form even somatic chimeras, let alone germ-line chimeras. An additional reason for introducing plasmid vectors into cell lines is that it opens up the possibility of targeted integration of DNA into the avian genome. Thus, by including a 1.8-kb fragment of the *ovalbumin* gene in their plasmid, Page *et al.* (1991) raised the possibility of using homologous recombination to get site-specific insertion. This is normally considered to be a relatively rare event in mammalian cells (1 in 10^4), but Buerstedde and Takeda (1991) obtained targeted integration in about 80% of transformed chick B cell lines. In this work constructs containing either chicken β-actin or *ovalbumin* genes were used. These results are extremely surprising, especially as they were not repeated in other cell lines such as T-cells, myoblasts or erythroblasts.

4.4.2.2 Cell insertion and chimera screening

The crucial factor determining the efficiency by which genetically manipulated cells are incorporated into a chimera is clearly the ratio of donor cells (C_d) to endogenous cells (C_e) for any specific tissue. If

AUG 21 '98 13:52 FR CISTI URGENT SERVICE TO 17033053585 P.25 34

totipotent blastoderm cells are used with no spatial preference for particular sites of embryo incorporation, the ratio obviously becomes simply one of relative cell numbers. In this situation Carscience *et al.* (1992) used a clever procedure of exposing recipient blastoderms to 540–660 rads of ^{60}Co radiation before adding donor cells. The effect of the radiation was to disrupt normal cell biology, so that the donor cells were at an advantage in populating the embryo. As a result the degree of chimerism increased by three- to four-fold. A general cellular disruption of this type is clearly the only approach to increasing efficiency when tissues have not differentiated, but once differentiation has progressed far enough to establish specific properties other possibilities exist.

A number of attempts have been made to remove PGCs in 2-day-old fowl embryos by surgical methods (McCarrey & Abbott 1982) or by destroying them with ultraviolet (Reynaud 1976), X-ray (Fargeix 1976) or laser (Mimms & McKinnell 1971) radiation. In all cases, however, it is difficult to avoid damage to surrounding tissues. An alternative approach is to use a chemosterilant such as the drug Busulphan (1,4-butanediol dimethane sulphonate), which destroys migrating PGCs in both mammals (Hemsworth & Jackson 1962) and birds (Reynaud 1981; Hallett & Wentworth 1991). This drug is a potential teratogen (Bishop & Wassom 1986), but by manipulating the dose and the route of uptake Aige-Gil and Simkiss (1991) were able to destroy 95% of the endogenous PGCs with minimal side effects. These approaches make it possible, therefore, to manipulate specifically the C_d/C_e ratio for germ-line cells.

In the formation of most chimeras, relatively little attention is paid to the sex of the donor and recipient cell lines. When chimeras are being used to form germ-line transgenics, however, there is clearly a potential problem. Thus, male PGCs (ZZ) inserted into a female embryo (WZ) or female PGCs (WZ) introduced into a male embryo (ZZ) may undergo abnormal processing in the developing gonad. All the evidence indicates that the type of gonad (i.e., ovary or testis) is determined by the genotype of the germinal ridge, but in quail–chick chimeras the stroma of the gonad was also able to harbour germ-cells of the opposite sex, at least for up to 12 days of incubation (Hajji *et al.* 1988). A somewhat different result was obtained by Haffen (1975), who made chimeras between anterior and posterior halves of chick embryos in culture before grafting them into coelomic sites. Under these conditions, male PGCs degenerated in ovaries, apparently be-

AUG 21 '98 13:52 FR CISTI URGENT SERVICE TO 17033053585 P.26/34

cause they could not cross the premeiotic prophase, while PGCs of either sex survived in the testis. There is, of course, a considerable difference in the onset of meiosis during the development of male and female gametes, and this is initiated during embryonic development (Dieterlen-Lievre *et al.* 1985). In the quail, it was possible to feminize male embryos so that the PGCs gave rise to oocytes. In the chick embryo, injections of an aromatase inhibitor (which blocks the synthesis of oestrogen from testosterone) caused females to develop testes and undergo full spermatogenesis (Elbrecht & Smith 1992). At present, no successful breeding experiments have been undertaken on such birds, so that it is not known if these gametes are functional. It is therefore not at all clear what will happen if mixed-sex chimeras are produced in attempts to produce germ-line chimeras.

4.4.3 Conclusions

The current indications are that the size of the avian oocyte, the deep abdominal location of the gonads and the extended time between fertilization and oviposition all militate against the efficient microinjection of the pronucleus as a way of producing transgenics. The alternative route towards this end is to produce some kind of chimera by either an *in ovo* or an *in vitro* manipulation of embryonic cells. This suggests the following considerations and problems:

1. Manipulation of blastoderm cells: To date this has been the most successful approach but with two constraints. These are (a) a dependence on retroviral vectors and (b) an inability to select for inserted DNA, resulting in great variability in offspring.
2. Manipulation of ES cells: This is by far the most intellectually exciting possibility, raising prospects of continual culture of pluripotent cells containing plasmids with selectable properties. Unfortunately, there are major constraints, starting with the need to identify true ES cells in the bird, extending to the problem of maintaining them in a nondifferentiating culture and culminating in the difficulty of ensuring efficient germ-line insertion.
3. Manipulation of PGCs: In many ways this is a compromise approach in that it initially was thought to depend on the use of committed cells that would enter the germ-line. Because of this commitment there are significant problems in inducing cell division in culture and ensuring that the PGCs are still capable of completing their migration to the gonad. There are major barriers to under-

AUG 21'98 13:53 FR CISTI URGENT SERVICE TO 17033053585 P.27

standing the membrane receptors that will control migration, proliferation and differentiation in the gonad, and the significance of this has recently been increased by the observation that pluripotent ES cells can be derived from PGCs (Matsui *et al.* 1992) and reinserted into the germ-line (Stewart *et al.* 1994).

In all three types of transgenesis the chimera approach depends on raising the embryo to a mature adult before it can be screened to see if the foreign DNA has entered the germ-line. Transgenics do not exist until the F1 generation, making the experiments expensive in time and resources. The probability of producing transgenic offspring from chimeric adults depends on the efficiency of introducing foreign DNA into the embryonic gonad, which in turn depends on the following:

4. Manipulation of the cell kinetics of the embryo in favour of the introduced transfected cells: In practice this involves disrupting either (a) the development of the whole embryo so that introduced cells are at an advantage in establishing themselves or (b) specific disruption (sterilization) of the germ cells so that the gonad is populated by transfected cells. This can be done physically (e.g., X rays, ultraviolet light, lasers) or chemically (e.g., chemosterilants). There is, however, a basic lack of information about the production and survival of germ cell clones in the vertebrate gonad.

At the present time (1992) the only vectors that have been successfully used to produce avian transgenics are retroviruses. There is a certain historical satisfaction about this since retroviruses were first discovered in chickens, but their instability and public image mean that plasmids may be more useful in the long term. Plasmid vectors have not been introduced into blastoderm cells *in ovo*. Thus, the most successful route for producing avian transgenics is not easily accessible to plasmid vectors without going down the cell chimera route.

Two other points are worth noting. First, relatively few genes have been isolated and cloned in the bird, so that current opportunities are limited (Bulfield 1990). Second, where avian genes have been successfully introduced into birds, as with *cGH*, the expected results were not obtained. The continued secretion of growth hormone down-regulates membrane receptors, and in order to get its normal physiological responses the hormone has to be pulsed (Johnson 1988; Bacon *et al.* 1989). It is somewhat paradoxical that the first efforts to produce

AUG 21'98 13:53 FR CISTI URGENT SERVICE TO 17033053585 P.28/34

avian transgenics turned out to be disappointing because the physiological properties of the protein that was produced were not fully appreciated. Clearly, the regulation of foreign DNA at the organ level will be an important priority in all transgenic work in the immediate future.

References

Aige-Gil, V. & Simkiss, K. (1991). Sterilisation of avian embryos with busulphan. *Res. Vet. Sci.* **50**, 139–44.

Al-Thani, R. & Simkiss, K. (1991). Effects of an acute in vivo application of concanavalin A on the migration of avian primordial germ cells. *Protoplasma* **161**, 52–7.

Bachiller, D., Schellander, K., Peli, J. & Ruther, U. (1991). Liposome-mediated DNA uptake by sperm cells. *Mol. Reprod. Dev.* **30**, 194–200.

Bacon, W. L., Vasilatos-Younken, R., Nestor, K. E., Anderson, B. L. & Long, D. W. (1989). Pulsatile patterns of plasma growth hormone in turkeys: Effects of growth rate, age and sex. *Gen. Comp. Endocrinol.*, **75**, 417–26.

Bellin, M. E., Gale, J. A. & Wentworth, B. C. (1985). Avian PGC growth in response to components of the extracellular matrix in vitro. *Biol. Reprod.* **32**(suppl.), 72 (abstract).

Bishop, J. B. & Wassom, J. S. (1986). Toxicological review of busulphan. *Mutation Res.* **168**, 15–45.

Bosselman, R. A., Hsu, R.-Y., Boggs, T., Hu, S., Bruszewski, J., Ou, S., Kozar, L., Martin, F., Green, C., Jacobsen, F., Nicholson, M., Schultz, J. A., Semon, K. M., Rishell, W. & Stewart, R. G. (1989a). Germ line transmission of exogenous genes in the chicken. *Science* **243**, 533–5.

Bosselman, R. A., Hsu, R.-Y., Boggs, T., Hu, S., Bruszewski, J., Ou, S., Souza, S., Kozar, L., Martin, F., Nicholson, M., Rishell, W., Schultz, J. A., Semon, K. M. & Stewart, R. G. (1989b). Replication-defective vectors of reticuloendotheliosis virus transduce exogenous genes into somatic stem cells of the unincubated chicken embryo. *J. Virol.* **63**, 2680–9.

Bosselman, R. A., Hsu, R.-Y., Briskin, M. J., Boggs, T., Hu, S., Nicholson, M., Souza, L. M., Schultz, J. A., Rishell, W. & Stewart, R. G. (1990). Transmission of exogenous genes into the chicken. *J. Reprod. Fert.* **41**,(suppl.), 183–95.

Brazolot, C. L., Petitte, J. N., Clark, M. E. & Etches, R. J. (1990). Introduction of lipofected chicken blastodermal cells into the early chicken embryo. *J. Cell Biochem. Suppl.* **15E**, 200 (abstract).

Brinster, R. L., Sandgren, E. P., Behringer, R. R. & Palmiter, R. D. (1989). No simple solution for making transgenic mice. *Cell* **59**, 239–41.

Buerstedde, J.-M. & Takeda, S. (1991). Increased ratio of targeted to random integration after transfection of chicken β cell lines. *Cell* **67**, 179–88.

Bulfield, G. (1990). Molecular genetics. In *Poultry Breeding and Genetics*, ed. R. D. Crawford, pp. 543–84. Amsterdam: Elsevier.

Burnstead, N. & Palyga, J. (1992). A preliminary linkage map of the chicken genome. *Genomics* **13**, 690–7.

Burnstead, N., Messer, L. L., Freeman, B. M. & Manning, A. C. C. (1987).

AUG 21 '98 13:53 FR CISTI URGENT SERVICE TO 17033053585 P.29

Genetic transformation of chickens using irradiated male gametes. *Heredity* **58**, 25–30.

Carscience, R. S., Clark, M. E. & Etches, R. J. (1992). Manipulation of the donor cell contribution to chimaeric chickens. *Poultry Sci.* **71**(suppl. 1), 72 (abstract).

Chen, H. Y., Garber, E. A., Mills, E., Smith, J., Kopchick, J. J., Dilella, A. G. & Smith, R. G. (1990). Vectors, promoters & expression of genes in chick embryos. *J. Reprod. Fert.* **41** (Suppl.), 173–82.

Climent, P. S., Gotzens, V. J. & Dominguez, L. (1979). Nuage in the primordial germ cells of the chick embryo. *Z. Vet. Med. C. Anat. Histol. Embryol.* **8**, 200–4.

Crittenden, L. B. & Salter, D. W. (1990). Expression of retroviral genes in transgenic chickens. *J. Reprod. Fert.* **40** (suppl.) 163–71.

Deeming, D. C., Rowlett, K. & Simkiss, K. (1987). Physical influences on embryo development. *J. Exp. Zool.* **1** (suppl.), 341–5.

Dieterlen-Lievre, F., Hajji, K. & Martin, C. (1985). Is the time of meiosis initiation programmed in the germ cells or the ovarian stroma? A study in avian chimaeras. *Arch. Anat. Microscop. Morphol. Exp.* **74**, 20–2.

Donovan, P. J., Stott, D., Cairns, L. A., Heasman, J. & Wylie, C. C. (1986). Migratory and post migratory mouse primordial germ cells behave differently in culture. *Cell* **44**, 831–8.

Elbrecht, A. & Smith, R. G. (1992). Aromatase enzyme activity and sex determination in chickens. *Science* **255**, 467–70.

Ephrussi, A. & Lehman, R. (1992). Induction of germ cell formation by oskar. *Nature* **358**, 387–92.

Etkins, L. D. & Pearman, B. (1987). Distribution, expression and germ line transmission of exogenous DNA sequences following microinjection into *Xenopus laevis* eggs. *Development* **26**, 194–202.

Evans, M. J. & Kaufman, M. H. (1981). Establishment in culture of pleuripotential cells from mouse embryos. *Nature* **292**, 154–6.

Eyal-Giladi, H. (1984). The gradual establishment of cell commitments during the early stages of chick development. *Cell. Diff.* **14**, 245–55.

Eyal-Giladi, H. & Kochav, S. (1976). From cleavage to primitive streak formation: A complementary normal table and a new look at the first stages of the development of the chick. 1. General Morphology. *Dev. Biol.* **49**, 321–37.

Eyal-Giladi, H., Debby, A. & Harel, N. (1992). The posterior section of the chick's area pellucida and its involvement in hypoblast and primitive streak formation. *Development* **116**, 819–30.

Fargeix, N. (1969). Les cellules germinales du canard chez des embryons normaux et des embryons de régulation: Etude des jeunes stades du developpement. *J. Embryol. Exp. Morphol.* **22**, 477–503.

Fargeix, N. (1986). Regulation du nombre des genocytes dans les ebauches gonadique de l'embryon de canard apres destruction partielle du stock initial des cellules germinales. *C.R. Acad. Sci. Paris.* **282D**, 305–8.

Ferhane-Tachinante, F. (1975). Experimental study of exchanges of germ cells in birds and mammals by culture and graft techniques. *Bull. Soc. Sci. Nat. Phys. Maroc.* **55**, 21–90.

Gardner, R. L. & Papaioannou, V. (1975). Differentiation in the trophectoderm and inner cell mass. In *The Early Development of Mammals*, ed. M. Balls & A. E. Wild, pp. 107–32. Cambridge University Press.

Gavora, J. S., Benkel, B., Sasada, H., Cantwell, W. J., Fiser, P., Teather,

AUG 21 '98 13:54 FR CISTI URGENT SERVICE TO 17033053585 P.30/34

R. M., Nagai, J. & Sabour, M. P. (1991). An attempt at sperm-mediated gene transfer in mice and chickens. *Can. J. Anim. Sci.* **71**, 287–91.

Ginsburg, M. & Eyal-Giladi, H. (1987). Primordial germ cells of the young chick blastoderm originate from the central zone of the area pellucida irrespective of the embryo-forming process. *Development* **101**, 209–19.

Ginsburg, M. & Eyal-Giladi, H. (1989). Primordial germ cell development in cultures of dispersed central discs of stage X chick blastoderms. *Gamete Res.* **23**, 421–8.

Gruenbaum, Y., Revel, E., Yarus, S. & Fainsod, A. (1991). Sperm cells as vectors for the generation of transgenic chickens. *J. Cell Biochem. Suppl.* **15E**, 194 (abstract).

Haffen, K. (1975). Sex determination of avian gonads in vitro. *Am. Zool.* **15**, 257–72.

Hajji, K., Martin, C., Perramon, A. & Dieterlen-Lievre, F. (1988). Sexual phenotype of avian chimaeric gonads with germinal and stromal cells of opposite genetic sexes. *Biol. Struct. Morphol.* **1**, 107–16.

Hallett, J. S. & Wentworth, B. C. (1991). The effect of busulphan on gonadal differentiation in Japanese quail (*Coturnix coturnix japonica*). *Poultry Sci.* **70**, 1618–23.

Hamburger, V. & Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morphol.* **88**, 49–92.

Hemsworth, B. N. & Jackson, H. (1962). Effect of busulphan on the foetal gonad. *Nature, Lond.*, **195**, 816–17.

Howarth, B. (1971). An examination of sperm capacitation in the fowl. *Biol. Reprod.* **3**, 338–41.

Hughes, G. C. (1963). The population of germ cells in the developing female chick. *J. Embryol. Exp. Morphol.* **11**, 513–36.

Hughes, G. C. (1964). The radiosensitivity of oogonia and oocytes in the chick embryo. *J. Embryol. Exp. Morphol.* **12**, 273–87.

Johnson, R. J. (1988). Diminution of pulsatile growth hormone secretion in the domestic fowl (*Gallus domesticus*): Evidence of sexual dimorphism. *J. Endocrinol.* **119**, 101–9.

Kopchick, J. J., Mills, E., Rosenblum, C., Taylor, J., Macken, F., Leung, F., Smith, J. & Chen, H. (1991). Methods for the introduction of recombinant DNA into chicken embryos. In *Transgenic Animals*, ed. N. L. First & F. P. Haseltine, pp. 275–93. Boston: Butterworth-Heinemann.

Kuwana, T., Maeda-Suga, H. & Fujimoto, T. (1986). Attraction of chick primordial germ cells by gonadal anlage *in vitro*. *Anat. Rec.* **215**, 403–6.

Lake, P. E. (1971). The male in reproduction. In *Physiology and Biochemistry of the Domestic Fowl*, ed. D. J. Bell, & B. M. Freeman, Vol. 3, pp. 1411–47. London: Academic Press.

Lavitrano, M., Camaioni, A., Fazio, V. M., Dolci, S., Farace, M. G. & Spadafora, C. (1989). Sperm cells as vectors of introducing foreign DNA into eggs: Genetic transformation of mice. *Cell* **57**, 717–23.

Le Douarin, N. & McLaren, A., eds. (1984). *Chimaeras in Developmental Biology*. London: Academic Press.

Lee, H., Karasany, N. & Nagele, R. G. (1978). The role of cell surface in the migration of primordial germ cells in early chick embryos: Effects of concanavalin A. *J. Embryol. Exp. Morphol.* **46**, 5–20.

Lee, M.-R. & Shuman, R. (1990). Transgenic quail produced by retrovirus vector infection transmit and express a foreign gene marker. *Proc. 4th World Congr. Genet. Appl. Livestock Prod.* **16**, 107–10.

AUG 21 '98 13:54 FR CIST URGENT SERVICE TO 17033053585 P.31 34

Lillie, F. R. (1919). *The Development of the Chick: An Introduction to Embryology*, 2d ed. New York: Holt.

Loveless, W., Bellairs, R., Thorpe, S. J., Page, M. & Feizi, T. (1990). Developmental patterning of the carbohydrate antigen FC102 during early embryogenesis in the chick. *Development* **108**, 97–106.

Marzullo, G. (1970). Production of chick chimaeras. *Nature, Lond.* **225**, 72–3.

Matsui, Y., Zsebo, K. & Hogan B. L. M. (1992). Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. *Cell* **70**, 841–7.

McCarrey J. R. & Abbott, U. K. (1982). Functional differentiation of chick gonads following depletion of primordial germ cells. *J. Embryol. Exp. Morphol.* **68**, 161–74.

McLaren, A. (1992). The quest for immortality. *Nature, Lond.* **359**, 482–3.

Meyer, D. B. (1964). The migration of primordial germ cells in the chick embryo. *Dev. Biol.* **10**, 154–90.

Meyers, N. L., Booth, S. C., Bumstead, N., Vick, L. & Simkiss, K. (1991). Development and testing of a packaging cell line for avian retroviral vectors. *Arch. Virol.* **119**, 257–64.

Mimms, M. F. & McKinnell, R. G. (1971). Laser irradiation of the chick embryo germinal crescent. *J. Embryol. Exp. Morphol.* **26**, 31–6.

Mongin, P. & Sauveur, B. (1970). Composition du fluide uterin et de l'albumen durant le sejour de l'oeuf dans l'uterus chez la poule domestique. *C. R. Acad. Sci. Paris* **270D**, 1715–18.

Motro, B., Kooy, D. Vander., Rossant, J., Reith, A. & Bernstein, A. (1991). Continuous patterns of ckit and steel expression: Analysis of mutation at the W & S1 loci. *Development* **173**, 1207–21.

Muniesa, P. & Dominguez, L. (1990). A morphological study of primordial germ cells at pregastrular stages in the chick embryo. *Cell Diff. Dev.* **31**, 105–17.

Naito, M., Nirasawa, K. & Oishi, T. (1990). Development in culture of the chick embryo from fertilized ovum to hatch. *J. Exp. Zool.* **254**, 322–6.

Naito, M., Agata, K., Otsuka, K., Kino, K., Ohta, M., Hirose K., Perry, M. M. & Eguchi, G. (1991a). Embryonic expression of β actin–lac Z hybrid gene injected into the fertilized ovum of the domestic fowl. *Int. J. Dev. Biol.* **35**, 69–75.

Naito, M., Watanabe, M., Kinutani, M., Nirasawa, K. & Oishi, T. (1991b). Production of quail-chick chimaeras by blastoderm cell transfer. *Br. Poultry Sci.* **32**, 79–86.

Nichols, J., Evans, E. P. & Smith, A. G. (1990). Establishment of germ-line competent embryonic stem (ES) cells using differentiating inhibiting factor. *Development* **110**, 1341–8.

Nieuwkoop, P. D. & Satasurya, L. A. (1979). *Primordial Germ Cells in the Chordates*. Cambridge University Press.

Ono, T. & Wakasugi, N. (1984). Mineral content of quail embryos cultured in mineral rich and mineral free conditions. *Poultry Sci.* **63**, 159–66.

Page, N., Savva, D., Luke, G. & Simkiss, K. (1991). Transfection of chick cells by non-retroviral DNA. *Biochem. Soc. Trans.* **19**, 328.

Palmiter, R. D. & Brinster, R. L. (1986). Germ-line transformation of mice. *Annu. Rev. Genet.* **20**, 465–99.

Pandey, K. K. & Patchell, M. R. (1982). Genetic transformation in chicken by the use of irradiated male gametes. *Mol. Gen. Genet.* **186**, 305–8.

Pardanaud, L., Altmann, C., Kitos, P., Dieterlen-Lievre, F. & Buck, C.

AUG 21 '98 13:55 FR CISTI URGENT SERVICE TO 17033053585 P.32 34

(1987a). Vasculogenesis in the early quail blastodisc as studied with a monoclonal antibody recognizing endothelial cells. *Development* 22, 47–60.

Pardanaud, L., Buck, C. & Dieterlen-Lievre, F. (1987b). Early germ cell segregation and distribution in the quail blastoderm. *Cell Diff.* 22, 47–60.

Perry, M. M. (1987). Nuclear events from fertilization to the early cleavage stages in the domestic fowl (*G. domesticus*). *J. Anat.* 150, 99–109.

Perry, M. M. (1988). A complete culture system for the chick embryo. *Nature, Lond.* 331, 70–2.

Perry, M. M., Morrice, D., Hettle, S. & Sang, H. (1991). Expression of exogenous DNA during the early development of the chick embryo. *Roux's Arch. Dev. Biol.* 200, 312–19.

Petitte, J. N., Clark, M. E., Luv, G., Gibbins, A. M. V. & Etches, R. J. (1990). Production of somatic and germ line chimaeras in the chicken by transfer of early blastodermal cells. *Development* 108, 185–95.

Petitte, J. N., Clark, M. E. & Etches, R. J. (1991). Assessment of functional gametes in chickens after transfer of primordial germ cells. *J. Reprod. Fert.* 92, 225–9.

Resnick, J. L., Bixler, L. S., Cheng, L. & Donovan, P. J. (1992). Long term proliferation of mouse primordial germ cells in culture. *Nature, Lond.* 359, 550–1.

Reynaud, G. (1969). Transfert de cellules germinales primordiales de Dindon à l'embryon de Poulet par injection iuntravasculaire. *J. Embryol. Exp. Morphol.* 21, 485–507.

Reynaud, G. (1976). Capacités reproductrices et descendance de poulets ayant subi un transfert de cellules germinales primordiales durant la vie embryonnaire. *Wilhelm Roux's Arch. Dev. Biol.* 179, 85–110.

Reynaud, G. (1981). The effect of busulfan on the germ cell line of the quail embryo. *Arch. Anat. Microscop. Morphol. Exp.* 70, 251–8.

Robertson, E. J. (1986). Pluripotential stem cell lines as a route into the mouse germ line. *Trends Genet.* 2, 9–13.

Rogulska, T., Ozdzenski, W. & Komar, A. (1971). Behaviour of mouse primordial germ cells in the chick embryo. *J. Embryol. Exp. Morphol.* 25, 155–64.

Romanoff, A. L. (1972). *Pathogenesis of the Avian Embryo*. New York: Wiley-Interscience.

Rosenblum, C. I. & Chen, H. Y. (1991). Liposome-mediated gene transfer into chicken embryos in ovo. *J. Cell. Biochem. Suppl.* 15E, 208 (abstract).

Rowlett, K. (1991). Embryo growth and development in culture. In *Avian Incubation*, ed. S. G. Tullett, pp. 107–24. Boston: Butterworth-Heinemann.

Rowlett, K. A. & Simkiss, K. (1985). The surrogate egg. *New Scientist*, 1469, 42–4.

Rowlett, K. & Simkiss, K. (1987). Explanted embryo culture: In vitro and in ovo techniques for domestic fowl. *Br. Poultry Sci.* 28, 91–101.

Salter, D. W., Smith, E. J., Hughes, S. H., Wright, S. E., Fadly, A. M., Witter, R. L. & Crittenden, L. B. (1986). Gene insertion into the chicken germ line by retroviruses. *Poultry Sci.* 65, 1445–58.

Salter, D. W., Smith, E. J., Hughes, S. H., Wright, S. E. & Crittenden, L. B. (1987). Transgenic chickens: Insertion of retroviral genes into the chicken germ line. *Virology*, 157, 236–40.

AUG 21 '98 13:55 FR CISTI URGENT SERVICE TO 17033053585 P.33/34

Sang, H. & Perry, M. M. (1989). Episomal replication of cloned DNA injected into the fertilized ovum of the hen *Gallus domesticus*. *Mol. Reprod. Dev.* **1**, 98–106.

Savva, D., Page, N., Vick, J. & Simkiss, K. (1991). Detection of foreign DNA in transgenic chicken embryos using the polymerase chain reaction. *Res. Vet. Sci.* **50**, 131–3.

Shih, C.-C., Stoye, J. P. & Coffin, J. M. (1988). Highly preferred targets for retroviral integration. *Cell* **53**, 531–7.

Shoffner, R. N., Otis, J. S. & Guise, K. (1987). Improbability of transfection by irradiated spermatozoa in the chicken. *Poultry Sci.* **66**(suppl.) 176 (abstract).

Shuman, R. M. (1986). Gene transfer in avian species using retrovirus vectors. *7th European Poultry Congress*, pp. 24–37.

Shuman, R. M. (1990). Genetic engineering. In *Poultry Breeding and Genetics*. ed. R. D. Crawford, pp. 585–98. Amsterdam: Elsevier.

Shuman, R. M. (1991). Production of transgenic birds. *Experientia* **47**, 897–905.

Simkiss, K. (1991). Primordial germ cells and the scope for genetic manipulation using embryos. In *Avian incubation*, ed. S. Tullett, pp. 125–36. London: Butterworth-Heinemann.

Simkiss, K. (1993). Surrogate eggs, chimaeric embryos & transgenic birds. *Comp. Biochem. Physiol.* **104A**, 411–17.

Simkiss, K., Rowlett, K., Bumstead, N. & Freeman, B. M. (1989). Transfer of primordial germ cell DNA between embryos. *Protoplasma* **151**, 164–6.

Simkiss, K., Vick, L., Luke, G., Page, N. & Savva, D. (1990). Infection of primordial germ cells with defective retrovirus and their transfer to the developing embryo. *4th World Congr. Genet. Appl. Livestock Prod.* **16**, 111–14.

Simkiss, K., Li, Y., Luke, G., Page, N. & Vick, L. (1993). The production of transgenic birds from primordial germ cell manipulated embryos. In *Avian Endocrinology*, ed. P. J. Sharp, pp. 367–80. Bristol: Journal of Endocrinology.

Simon, D. (1960). Contribution à l'étude de la circulation et du transport des gonocytes primaires dans les blastodermes d'oiseau cultivés in vitro. *Arch. Anat. Microsc. Morphol. Exp.* **49**, 93–176.

Spencer, J. L., Gavora, J. S. & Gowe, R. S. (1980). Lymphoid leukosis virus: Natural transmission and non-neoplastic effects. *Proc. Cold Spring Harbor Conf. Cell Prolif.* **7**, 553–64.

Spratt, N. T. & Haas, H. (1960). Morphogenetic movements in the lower surface of the incubated and early chick blastoderm. *J. Exp. Zool.*, **144**, 139–57.

Stern, C. D. (1990). The marginal zone and its contribution to the hypoblast and primitive streak of the chick embryo. *Development* **109**, 667–82.

Stewart, C. L., Gadi, I. & Bhatt, X. (1994). Stem cells from primordial germ cells can reenter the germ line. *Dev. Biol.* **161**, 626–8.

Temin, H. M. (1989). Retrovirus vectors, promise and reality. *Science* **246**, 983.

Thomas, K. R. & Capecchi, M. R. (1987). Site-directed mutagenesis by gene targeting in mouse embryo derived stem cells. *Cell* **51**, 503–12.

Tomita, T., Yamamoto, N., Otsuka, K., Ohta, M. & Hirose, K. (1988). Genetic transformation of eggshell colour in chickens by the use of irradi-

AUG 21'98 13:56 FR CISTI URGENT SERVICE TO 17003053585 P.034 34

ated sperm. In *Proceedings of the 18th World Poultry Congress, Nagoya, Japan*, pp. 515–16.

Ukeshima, A. & Fujimoto, T. (1984). Ultrastructure of primordial germ cells in the early chick embryo. In *Ultrastructure of Reproduction*, ed. J. Van Blerkom & P. M. Motta, pp. 12–18. Boston: Martinus Nijhoff.

Urven, L. E., Erickson, C. A., Abbott, U. K. & McCarrey, J. R. (1988). Analysis of germ line development in the chick embryo using an anti-mouse EC cell antibody. *Development* **103**, 299–304.

Varmus, H. (1988). Retroviruses. *Science*. **240**, 1427–35.

Vick, L., Li, Y. & Simkiss, K. (1993). Transgenic birds from transformed PGCs. *Proc Roy. Soc. Lond.* **251**, 179–82.

Watanabe, S. & Temin, H. M. (1983). Construction of a helper cell line for avian reticulo endotheliosis virus cloning vectors. *Mol. Cell Biol.* **3**, 2241–9.

Wentworth, A. L., Frank, A. & Wentworth, B. C. (1992). Transfection of Japanese quail primordial germ cells with DNA expressing gene product lac Z. *Poultry Sci.*, **71**(Suppl. 1), 73 (abstract).

Wentworth, B. C. & Wentworth, A. (1991). Primordial germ cell characteristics in the Japanese quail embryo. *Poultry Sci.*, **70**(Suppl. 1), 129 (abstract).

Wentworth, B. C., Tsai, H., Hallett, J. H., Gonzales, D. S. & Rajcic-Spasojevic, G. (1989). Manipulation of avian primordial germ cells and gonadal differentiation. *Poultry Sci.* **68**, 999–1010.

Witte, O. N. (1990). Steel locus defines new multipotent growth factor. *Cell.* **63**, 5–6.

Yoshinaga, K., Fujimoto, T., Nakamura, M. & Terakura, H. (1992). Selective lectin-building sites of primordial germ cells in chick and quail embryos. *Anat. Rec.* **233**, 625–32.

** TOTAL PAGE.034 **